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Assessment of the Mutagenic Potential of Carbon Disulfide, Carbon Tetrachloride, Dichloromethane, Ethylene Dichloride, and Methyl Bromide: A Comparative Analysis in Relation to Ethylene Dibromide

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ASSESSMENT OF THE MUTAGENIC POTENTIAL OF CARBON DISULFIDE, CARBON TETRACHLORIDE, DICHLOROMETHANE, ETHYLENE DICHLORIDE, AND METHYL BROMIDE: A COMPARATIVE ANALYSIS IN RELATION TO ETHYLENE DIBROMIDE

by

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16. ABSTRACT

This document provides an evaluaton of the mutagenic potential of five lternative fumigants to ethylene dibromide(EDB). These include carbon disulfide(CS_2), arbon tetrachloride(CCl_4), dichloromethane(DCM), ethylene dichloride(EDC), and methyl romide(MB). Of the five proposed alternatives, DCM, EDC, and MB caused gene mutations n lower and higher eucaryotes. These three agents have not been well studied for heir potential to cause chromosome damage. There are no available data on the ability f these agents to cause gene mutations in whole mammals, except for EDC, for which the vidence is suggestive. The potential of the alternatives to reach whole mammal germ-ell DNA has not been studied. But DCM, EDC, and MB do cause heritable effects in rosophila. CS_2 and CCl_4 have been predominately negative in mutagenicity tests. dditional studies are needed before a final judgment is reached with respect to their utagenic potential, however. The mutagenic potencies of each agent were compared ithin and among various assay systems. Regarding the strength of mutagenicity, EDB is learly a stronger mutagen than any of the proposed alternative fumigants. Differences n mutagenicity potency among the alternatives cannot be delineated because of limited ata usually derived from different laboratories. When final judgments could not be eached because of gaps in current knowledge, recommendations were made on additional tudies that could be conducted to better define the potential mutagenic risk.

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PREFACE

The Reproductive Effects Assessment Group was requested by the Hazard Evaluation Division of the Office of Pesticide Programs (OPP) to prepare a mutagenicity assessment of proposed pesticide alternatives to the fumigant, ethylene dibromide. These alternatives included carbon disulfide, carbon tetrachloride, dichloromethane, ethylene dichloride, and methyl bromide. This mutagenicity assessment is to serve as a "source document" for OPP's use.

In the development of this document, the scientific literature has been inventoried, and key studies have been critically evaluated. The Environmental Mutagen, Carcinogen, and Teratogen Information Department at the Oak Ridge National Laboratory identified the published literature.

Three sections of chapter 4 in this document have been taken from the health assessment documents prepared by the Office of Health and Environmental Assessment (OHEA) for the Office of Air Quality Planning and Standards. These sections include data evaluations of carbon tetrachloride, dichloromethane, and ethylene dichloride. The Health Assessment Document for Carbon Tetrachloride has received full administrative and peer review and is being submitted to the National Technical Information Service for publication. The Health Assessment Document for Dichloromethane and the Health Assessment Document for Ethylene Dichloride are still draft documents that are undergoing public review and comment and EPA Science Advisory Board review. The reader is referred to the health assessment documents (U.S. EPA, 1983a, 1984b, 1984c), if additional information is needed regarding health effects other than mutagenicity or background information such as physical-chemical properties.

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1. SUMMARY

One objective of this mutagenicity evaluation is to determine if five proposed alternatives to ethylene dibromide have the potential to cause mutations in humans. If mutations occur in germ cells, they may be passed on to future generations and increase the incidence of genetic disease in the human population; if mutations occur in somatic cells, they may lead to the onset of cancer and possibly other diseases. A second objective is to determine whether there are differences in the types of genetic damage induced or in the potency of mutagenic activity among these proposed alternatives.

Of the five proposed alternatives, three agents (dichloromethane, ethylene dichloride, and methyl bromide) cause gene mutations in several diverse organisms ranging from bacteria to mammalian cells in culture. These agents have not been sufficiently studied for their potential to cause chromosomal aberrations. Except for ethylene dichloride, there are no available data on the ability of these agents to cause gene mutations in whole mammals. The data for ethylene dichloride are limited, but they suggest the induction of mutation in somatic cells. The potential of the alternative fumigants to reach germ-cell DNA in intact mammals has not been sufficiently studied, but dichloromethane, ethylene dichloride, and methyl bromide do cause heritable effects in male Drosophila. Results of carbon disulfide and carbon tetrachloride in mutagenicity tests have been predominately negative, but additional studies are needed before a definitive judgment is reached with respect to their mutagenic potential.

Ethylene dibromide is a stronger mutagen than its structural analog, ethylene dichloride, in several different organisms (bacteria, Drosophila, mammalian cells in culture). This finding is in keeping with the relative electrophilicities of bromine and chlorine atoms. Ethylene dibromide also appears to be a stronger mutagen than dichloromethane and methyl bromide, but this conclusion is based on limited information. Differences among the alternatives cannot be readily delineated because their mutagenic activities generally fall within the same order of magnitude and this was further complicated by the limited data available and the lack of comparability in studies from different laboratories.

2. INTRODUCTION

This document provides an evaluation of the mutagenic potential of five proposed alternatives to the use of the fumigant, ethylene dibromide. The alternative compounds are carbon disulfide, carbon tetrachloride, dichloromethane, ethylene dichloride, and methyl bromide (Figure 2-1). The evaluation involved a survey and critical analysis of relevant studies. A separate analysis of the mutagenicity of each proposed alternative is found in the individual sections of Chapter 4. The evaluation of the five proposed alternative fumigants included a determination of the intrinsic mutagenic potential of each agent and its ability to reach germinal tissue in intact mammals. Ethylene dibromide is not included as a separate section because it has been evaluated previously by OPP.

A comparative analysis of mutagenicity between each of the proposed alternatives and ethylene dibromide is presented. The spectrum of genetic damage induced by each agent is discussed and mutagenic potencies are compared whenever appropriate. Because judgments can not be reached due to gaps in current knowledge, recommendations are made for additional studies that could be conducted to determine if a potential mutagenic risk exists.

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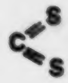
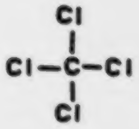
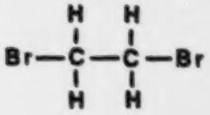
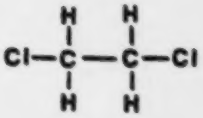
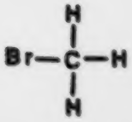
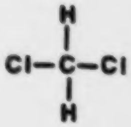
CARBON DISULFIDE	
CARBON TETRACHLORIDE	
ETHYLENE DIBROMIDE (1,2-DIBROMOETHANE)	
ETHYLENE DICHLORIDE (1,2-DICHLOROETHANE)	
METHYL BROMIDE (BROMOMETHANE)	
METHYLENE CHLORIDE (DICHLOROMETHANE)	

Figure 2-1. Chemical structures of ethylene dibromide and proposed alternatives.

3. COMPARATIVE ANALYSIS OF ETHYLENE DIBROMIDE AND PROPOSED ALTERNATIVES

In this chapter, data on the mutagenicity of the five proposed alternative fumigants are reviewed and compared with data on ethylene dibromide. The comparisons consider the spectrum of genetic damage that is induced by each agent and mutagenic potencies in selected tests.

3.1. QUALITATIVE

The evaluation of data to judge whether a chemical is mutagenic and has the potential to cause mutations in human germ cells can be referred to as a qualitative assessment. A qualitative assessment uses a weight-of-evidence approach that considers such factors as the types of genetic damage induced (e.g., gene mutation, structural or numerical chromosome mutation), the type and number of tests used, and the quality and adequacy of the testing. The U.S. Environmental Protection Agency's (EPA) Proposed Mutagenicity Risk Assessment Guidelines (U.S. EPA 1980, 1984d) considers, for example, that two positive gene mutation tests in phylogenetically different organisms (e.g., mammalian cells in vitro and bacteria) provide sufficient evidence to judge a chemical to be mutagenic. When such evidence is coupled with data from whole mammals regarding the ability of the agent to reach germ-cell DNA, human germ-cell mutagenicity is presumed. This approach is consistent with that outlined by the National Academy of Sciences (NAS) Committee on Chemical Environmental Mutagens (NAS, 1982).

3.1.1. Gene Mutation

Mutagenic endpoints that have been evaluated for the proposed alternative fumigants in various organisms and the numbers of studies conducted are listed in Table 3-1. Information from abstracts or review articles that do not provide original data are not included. Based on the evaluations in Chapter 4, the results were designated as "+" for a positive result, "-" for a negative result,

TABLE 3-1. QUALITATIVE COMPARISON OF THE MUTAGENICITY OF ETHYLENE DIBROMIDE AND PROPOSED ALTERNATIVES [a,b,c]

Chemical	Gene mutation						Clastogenicity		Numerical chromosome mutation	Other indicators of DNA damage[d]	DNA binding
	Bacteria	Fungi	Higher Plants	Drosophila	Mammalian cells	Whole mammals	in vitro	in vivo			
Carbon disulfide	-(1) I(3)			I(2)				W(1) -(2)	I(2)	I(1), UDS	
Carbon tetrachloride	W(1) I(4) -(2)	W(1)					-(1)			W(1), YMR -(3), UDS	+(2)
Dichloromethane	+(12) I(2)	+(1)		+(1) I(1)	I(1)		+(1)	W(1) -(1)		(I), YMR (+), YMR W(2), SCE -(2), UDS	
Ethylene dibromide	+(5) -(4)	+(3)	+(3)	+(3)	+(2)		+(1)	+(2)[e] -(2)		+(1), YMR +(1), UDS -(1), UDS +(1), Pol +(1), SCE	+(1)
Ethylene dichloride	W(7)[f] I(3)		+(1) I(1)	+(4)	+(2)	W(1)		-(2)	+(1)[g] W(1)[g]	I(1), UDS I(1), Pol	+(1)
Methyl bromide	+(4)			+(1) I(1)	+(1)			I(2)		I(2), UDS	+(1)

[a] The results are based on the evaluations presented in chapter 4 of this document. Information on ethylene dibromide is based on OPP documents (U.S. EPA, 1983a; Mauer, 1979; Lee, 1980).

[b] "+" designates a positive result; "-" a negative result; "I" an inconclusive study; "W" a weak, borderline, or suggestive result.

[c] Numbers of studies are indicated in parentheses; abstracts and review articles are not included.

[d] UDS = unscheduled DNA synthesis; YMR = yeast mitotic recombination; SCE = sister chromatid exchange; Pol = bacteria Pol A assay

[e] Plants only.

[f] Stronger response with an S9 metabolic activation system.

[g] Drosophila only.

"W" for a weak or suggestive response, and "I" for an inconclusive study. The information pertaining to the mutagenicity of ethylene dibromide was derived from OPP's documents (U.S. EPA, 1983a; Mauer, 1979; Lee, 1980). Similar to ethylene dibromide, the compounds dichloromethane, ethylene dichloride, and methyl bromide cause gene mutations in two or more unrelated organisms (see Table 3-1). Ethylene dibromide is active in a wide range of organisms; bacteria, Drosophila, fungi, mammalian cells, and higher plants (for review see Mauer, 1979 and Rannug, 1980). Dichloromethane, ethylene dichloride, and methyl bromide are mutagenic in bacteria and Drosophila (see chapter 4). Dichloromethane is also mutagenic in yeast (Callen et al., 1980), and ethylene dichloride is active in plants (Ehrenberg et al., 1974). Ethylene dichloride and methyl bromide are both mutagenic in mammalian cells in culture (Tan and Hsie, 1981; Gentese Limited Partnership, 1984; Kramers et al., 1984), whereas dichloromethane has not been adequately evaluated. Ethylene dichloride is the only agent for which results are available on effects in whole mammals (the mouse spot test: Gocke et al., 1983); the findings are suggestive of a positive response. Carbon disulfide and carbon tetrachloride do not appear to cause gene mutations, but available data are not sufficient to support definitive conclusions as can be seen in Table 3-1.

In Drosophila, there are differences among the compounds in the pattern of germ-cell stage sensitivity. The mutagenic effects of ethylene dibromide and its structural analog, ethylene dichloride, are greatest in spermatogonia, spermatocytes or spermatids (Kale and Baum, 1979; King et al., 1979), whereas the postmeiotic germ-cell stages appear to be most sensitive to methyl bromide and dichloromethane (Kramers et al., 1984; Gocke et al., 1981). The higher frequency of lethals in mature sperm and lower frequencies in earlier germ-cell stages may suggest that methyl bromide and dichloromethane are repaired efficiently or that labile DNA adducts are formed. On the other hand, there is evidence from the Drosophila sex-linked recessive lethal test and DNA alkylation studies that ethylene dibromide forms stable adducts that are apparently not efficiently repaired in testicular DNA (Lee, 1980). Thus, ethylene dibromide-induced damage would be expected to accumulate in germ cells. It is unknown whether the structural analog, ethylene dichloride, forms stable adducts. DNA binding studies using different dosage levels and sampling times are needed to determine whether or not stable adducts are formed after dichloromethane, ethylene dichloride, or methyl bromide exposure. Such data are an important issue because a mutagenic agent producing damage that accumulates might be expected to pose a greater hazard than one producing damage that forms labile adducts or is repaired efficiently.

In bacterial tests, ethylene dibromide and the alternative fumigants, dichloromethane*, ethylene dichloride, and methyl bromide, do not require an exogenous source of metabolic activation for the induction of mutagenicity (see Barber et al., 1981; Moriya et al., 1983; Jongen et al., 1978; Green, 1980; Snow et al., 1979). Although S9 activation has no enhancing effect on the mutagenic response of methyl bromide, it enhances the responses of dichloromethane, ethylene dibromide, and ethylene dichloride. Therefore, all of these agents are direct-acting mutagens with the latter three compounds apparently metabolized to stronger mutagenic intermediates.

Dichloromethane undergoes oxidative dechlorination by the microsomal P-450 mixed-function oxidase system, and formyl chloride is believed to be an intermediate in this pathway (U.S. EPA, 1983b). A second pathway involves a glutathione transferase system which dehalogenates dichloromethane to produce formaldehyde (a known mutagen), which is further oxidized to carbon dioxide. S-chloromethyl glutathione is thought to be an intermediate in this pathway. Although both formyl chloride and S-chloromethyl glutathione are highly unstable, they are very reactive alkylating agents. Thus, they are likely to be mutagenic if they reach DNA. At least two metabolic pathways also exist for both ethylene dibromide and ethylene dichloride (Rannug, 1980). One involves the microsomal mixed-function oxidases producing a haloacetaldehyde, and the other involves conjugation with glutathione, giving rise to a highly reactive half sulfur-mustard [S-(2-haloethyl)-L-cysteine]. Both of these metabolites are likely to be strong bacterial mutagens. Methyl bromide is an alkylating agent that directly reacts with nucleophilic sites in DNA. Although carbon tetrachloride has been predominantly negative in bacterial tests, it is expected to produce the free radical $\cdot CCl_3$ and phosgene (U.S. EPA, 1984a). There is insufficient information regarding the mutagenic potential of phosgene (U.S. EPA, 1984b). It is possible, however, that it is too short-lived to produce a detectable effect.

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*Metabolism by the bacteria is occurring; see section 4.3. for discussion.

3.1.2. Chromosome Mutation

The ability of ethylene dibromide and the proposed alternative fumigants to cause chromosomal damage have not been sufficiently studied. Among the few studies on ethylene dibromide, those in whole mammals have been negative, (Epstein et al., 1972; Generoso, 1978), whereas positive results have been reported in cultured mammalian cells (Tezuka et al., 1980), and positive and negative results have been reported implants (Ma et al., 1978; Ehrenberg et al., 1974). Two negative tests in whole mammals (which are considered inconclusive) are reported for methyl bromide (McGregor, 1981), and there is a single negative test of carbon tetrachloride in cultured mammalian cells (Dean and Hodson-Walker, 1979). Dichloromethane is positive in cultured mammalian cells (Thilager and Kumaroo, 1983) and there is a micronucleus test that suggests that it also induces chromosomal damage in intact mammals (Gocke et al., 1981). Only two studies in whole mammals were available for ethylene dichloride, and both were negative (King et al., 1979; Jenssen and Ramel, 1980): ethylene dichloride, however, has been shown to cause meiotic nondisjunction (i.e., numerical chromosome mutations) in Drosophila (Shakarnis, 1970). Except for carbon disulfide which gave results that are merely suggestive of a positive response (Vasil'eva, 1982), the other agents have not been evaluated for nondisjunction.

The available results on the capacity of these chemicals to cause chromosomal damage are inadequate to draw clear comparisons. The available data are not extensive and in some cases appropriate exposure levels and concurrent controls were not used. Based upon these limited data, it appears that none of these agents is a strong clastogen.

3.1.3. Other Indicators

Ethylene dibromide and some of the proposed alternatives have been evaluated using other indicators of DNA damage. Ethylene dibromide has been reported to induce sister chromatid exchange in mammalian cells in vitro, mitotic recombination in yeast and differential growth inhibition of DNA repair-deficient and repair-proficient strains of bacteria (i.e., pol A assay) (Tezuka et al., 1980; Fahrig, 1974; Brem et al., 1974). The induction of unscheduled DNA synthesis (UDS) has been reported for mammalian lymphocytes in vitro (Meneghini, 1974), but negative findings are reported for mammalian germ cells in vivo (Lee, 1980). The structural analog, ethylene dichloride, was reported to produce positive results in the bacterial pol A assay (Brem et al., 1974) and in a UDS assay using mammalian cells in vitro (Perocco and Prodi, 1981) but these data are equivocal. Dichloromethane has been shown not to stimulate UDS (Jongen et al., 1981; Perocco and Prodi, 1981) but produces a weak increase in sister chromatid exchanges in mammalian cells in vitro (Jongen et al., 1981; Thilagar and Kumaroo,

1983) and induces mitotic recombination in yeast (Callen et al., 1980). Carbon tetrachloride has not been adequately evaluated for other indicators of DNA damage; there are positive results for the induction of mitotic recombination in yeast (Callen et al., 1980) and negative results for the induction of UDS in mammalian systems (Mirsalis et al., 1982; Mirsalis and Buttersworth, 1980; Craddock and Henderson, 1978). Tests of methyl bromide and carbon disulfide for the induction of UDS in mammalian cells in vitro are reported as negative (McGregor, 1981; Kramers et al., 1984; Beliles, 1980) but are not considered conclusive.

In summary, ethylene dibromide, dichloromethane, and ethylene dichloride have been shown to be positive in tests for other indicators of DNA damage, albeit the data are limited. It is not clear whether the five proposed alternative fumigants elicit UDS. It should be stressed that certain kinds of DNA alterations that lead to mutations may not stimulate UDS or may do so to such a small extent (e.g., short patch repair) that UDS detection is not practical.

3.1.4. DNA Alkylation

Ethylene dibromide, dichloromethane, ethylene dichloride, and methyl bromide are alkylating agents. As shown in Table 3-1, three of the proposed alternative fumigants to ethylene dibromide have been shown to form adducts in DNA. Ethylene dibromide alkylates both mammalian germ- and somatic-cell DNA in vivo and Drosophila germ-cell DNA (Lee, 1980); for methyl bromide and ethylene dichloride, only mammalian somatic tissues (liver, spleen, kidney) have been studied (Reitz et al., 1982; Djalali-Behzad et al., 1981). Although carbon tetrachloride has not been demonstrated to be mutagenic, it has been found to bind to DNA in mammals (Rocchi et al., 1973; Diaz Gomez and Castro, 1980a). No studies are currently available for dichloromethane. The degree of alkylating activity exhibited by these agents will be discussed later.

3.1.5. Summary and Conclusions

Of the five proposed alternative fumigants, there is sufficient evidence on ethylene dichloride, dichloromethane, and methyl bromide, in addition to ethylene dibromide itself, to classify them as mutagens (Table 3-2). These chemicals have been reported as positive in two or more gene mutation tests in phylogenetically different organisms (see Table 3-1). There is also ancillary information regarding their DNA-damaging potential (e.g., SCE, DNA repair, DNA alkylation). The evidence that ethylene dichloride is a presumed mammalian mutagen is stronger than that for dichloromethane or methyl bromide because of (1) the larger number of positive tests conducted in different laboratories, (2) the suggestive evidence that ethylene dichloride causes somatic gene mutations in whole mammals, and (3) a study demonstrating the alkylation of DNA in somatic tissues of whole mammals. Although the data on the ability of these agents to cause chromosomal aberrations are limited, none of the chemicals appear to be strong clastogens. It is uncertain whether these agents produce a similar array of other types of genetic damage (e.g., nondisjunction, SCEs, mitotic recombination), because they have not all been sufficiently evaluated for the induction of other types of genetic alterations. Furthermore, it is uncertain whether the proposed alternatives reach and interact with mammalian germ-cell DNA, but ethylene dibromide is known to do so and this is presumed to be a human germ-cell mutagen. Ethylene dichloride, methyl bromide, and dichloromethane are positive in the Drosophila sex-linked recessive lethal test. This test organism has germ-cell stages analogous to those in mammals and provides some information regarding germ-cell risk in intact animals. Although the data bases are not equally complete for each of the compounds, none of the proposed alternatives appear to be as mutagenic as ethylene dibromide

TABLE 3-2. MUTAGENIC POTENTIAL OF ETHYLENE DIBROMIDE AND PROPOSED ALTERNATIVES

Mutagenic Activity	Chemical
Presumed human germ-cell mutagen	Ethylene dibromide[a]
Confirmed mutagenic activity but insufficient information on chemical interaction with mammalian germ-cell DNA[b]	Dichloromethane Ethylene dichlorine Methyl bromide
Insufficient information to reach any judgment[c]	Carbon disulfide Carbon tetrachloride

[a] Evidence of DNA binding in whole mammal germ cells.

[b] No data on ability to reach mammalian germinal tissue, but these chemicals have effects on germ cells in Drosophila.

[c] Available studies for these chemicals generally negative or weakly positive.

when results from similar tests are compared. (Mutagenic potency is discussed later in this chapter in section 3.2).

The mutagenic potential of the other two alternatives, carbon disulfide and carbon tetrachloride, could not be judged because of insufficient information. The available studies suggest, however, that if they are mutagenic, they are weakly so. This conclusion does not necessarily apply to chromosome nondisjunction because carbon tetrachloride has not been evaluated for its ability to disrupt spindle structures or function, and inadequate evidence is available for the induction of numerical chromosomal aberrations by carbon disulfide. The reader is referred to chapter 4 of this document for a critical analysis of the data pertaining to the mutagenicity of these five proposed alternative fumigants and for a detailed summary for each chemical.

3.2. MUTAGENIC POTENCIES

The mutagenic potencies of each of the five proposed alternative fumigants were compared with those of ethylene dibromide using results from the Salmonella assay. This test was the only one in which all chemicals have been evaluated. Several criteria were imposed to select appropriate experiments for this analysis. Only experiments using the desiccator procedure were included because all of the chemicals are volatile, and testing in sealed containers is more appropriate than in the standard plate assay in which the volatile test material evaporates and escapes. In addition, results were considered only if there were at least two nonzero dose points; spontaneous counts were reported, and revertant data were given in the report. Results on tester strain TA100 in the presence or absence of metabolic activation were used because this was the only strain for which data were available on all the compounds. A simple linear regression analysis was used on the linear portion of the dose-responses; linear regression calculations with correlation coefficients less than 0.90 were not accepted.

TABLE 3-3. DOUBLING DOSE OF THE PROPOSED ALTERNATIVES AND ETHYLENE DIBROMIDE IN THE SALMONELLA TA100 USING A DESSICATOR PROCEDURE

Chemical	Duration of exposure (hours)	Doubling dose (ug)[a] +S9	-S9	Reference[b]
A.[c] Ethylene dibromide	48	93 (Aroclor rat)	83	Barber et al., 1981
Ethylene dichloride	48	7286 (Aroclor rat)	8296	Barber et al., 1981
Dichloromethane	48	961 (Aroclor rat)	1641	Barber et al., 1980
B.[d] Dichloromethane	7	---	2.8	Simmon, 1978
	not reported	0.2 (DCM hamster)	0.95	Snow et al., 1979
	up to 72	267 (Aroclor rat)	357	Green, 1980
	up to 72	1012 (Aroclor rat)	1301	Green, 1981
	48	748 (Aroclor rat)	---	Jongen et al., 1980
	48	587 (Phenobartital rat)	872	Jongen et al., 1978
Methyl bromide	21	---	2.4	Simmon, 1978

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[a] Slope of dose response determined by simple linear regression analysis except for ethylene dibromide where slope was calculated by the following equation $\frac{[XY]}{[X^2]}$

[b] Discussion of these references can be found in chapter 4 for each proposed alternative fumigant.

[c] Agar concentration of test chemical measured by GC/MS in aqueous phase of petri dish.

[d] Agar concentration of test chemical was based on theoretical calculations of concentration of test agent in the gas phase: Concentration (umole) in agar = volume of gas in dessicator x solubility in agar x 760/vapor presssure of test chemical at solubility x 10^6/MW x density of agar x amount of agar added per plate.

As shown in Table 3-3, the doubling dose was then determined for each chemical (i.e., the dose producing a twofold increase in the spontaneous frequency). When studies are compared from the same laboratory (Barber et al., 1980; 1981) ethylene dibromide is a stronger mutagen than ethylene dichloride and dichloromethane (Table 3-3A). Although the reported mutagenic activities for ethylene dichloride and dichloromethane overlap and fall within one order of magnitude, it appears that dichloromethane is slightly more active than ethylene dichloride. It should be cautioned that although these studies were performed by the same laboratory group, they were not done concurrently, and thus technical and biological variation could account for the differences. It does appear, however, that these proposed agents are weak mutagens when considering the amount of material needed to cause a doubling in the spontaneous mutation frequency. Simmon (1978) concurrently tested methyl bromide, dichloromethane, and carbon tetrachloride in desiccators using Salmonella TA100 without S9 activation. Carbon tetrachloride was reported as negative under conditions which elicited positive responses for methyl bromide and dichloromethane. It should be pointed out that the exposure time was much longer for methyl bromide (21 hours) than for dichloromethane (7 hours), and testing was conducted only in the absence of S9. Other studies used to develop the data in Table 3-3B were not performed in the same laboratory, and as can be seen for dichloromethane, a difference in doubling dose ranged from 0.95 to 1301 ug with S9 and 0.2 to 1012 ug without S9. Therefore, interlaboratory variations can account for the observed differences (e.g., type of S9 and length of exposure times varied).

It is clear that ethylene dibromide is a much more potent mutagen than ethylene dichloride and dichloromethane. This is not surprising because brominated compounds are generally more mutagenic than the corresponding chlorinated compounds (Rannug, 1980). Although there are no data from the same laboratory, ethylene dibromide is expected to be more mutagenic than methyl bromide based on structural-activity relationships. Ethylene dibromide is a bi-functional agent, which are generally more biologically reactive. Carbon tetrachloride and carbon disulfide are predominantly negative in bacterial tests.

Potencies were also examined in the Drosophila sex-linked recessive lethal test (Table 3-4). Data were available for ethylene dibromide, ethylene dichloride, dichloromethane, and methyl bromide. Although certain germ-cell stages appear to be more sensitive than others, the total lethal frequencies were compared. As can be seen in Table 3-4, ethylene dibromide is a more potent mutagen than the alternatives, whether data from inhalation or feeding experiments are compared (Table 3-4). It is uncertain if ethylene dichloride is more active than methyl bromide. There is some overlap of the lethal frequencies per unit of exposure for these alternatives. Different strains of Drosophila were used, which could account for differences in lethal frequencies. Although carbon disulfide has been reported as negative, the possibility of weak effects cannot be excluded. In feeding experiments, ethylene dichloride seems to be more active than dichloromethane. However, these experiments were also conducted in different laboratories, and factors such as stocks of Drosophila and solvents differed; these variations could contribute observed differences among the alternatives in lethal frequencies. Nevertheless, in their totality, the data show that ethylene dibromide is more mutagenic than the proposed alternatives in the Drosophila sex-linked recessive lethal test. The Drosophila results are therefore consistent with those in Salmonella.

The mutagenicity of the alternative compounds in cultured mammalian cells cannot readily be compared to that of ethylene dibromide because test results

TABLE 3-4. MUTAGENIC ACTIVITY OF ETHYLENE DIBROMIDE AND CERTAIN PROPOSED ALTERNATIVES IN THE DROSOPHILA SEX-LINKED RECESSIVE LETHAL TEST

Chemical	Strain	Dose (ppm·h)	Ratio of lethals (% treated/% control)	References[a]
A. Inhalation Exposure				
Ethylene dibromide	Canton-S	125	16	Kale and Baum, 1979
Ethylene dichloride:	Canton-S and D-32	2,800	11	Shakarnis, 1969 and 1970
		2,800	40	
		4,200	66	
		5,600	20	
Methyl bromide	Berlin-K	3,446	14	Kramers et al., 1984, unpublished
		2,653	8	
		2,031	2.2	
		1,415	3.6	
		1,061	1.4	
Carbon disulfide	Not reported	280 19,200 (highest ineffective dose)		Beliles et al., 1980; Donner et al., 1981
		Dose (mM)		
B. Feeding Exposure				
Ethylene dibromide	Berlin-K	2.7 (507 ppm)	6	Vogel and Chandler, 1974
		5.4 (1014 ppm)	5	
Ethylene dichloride	Berlin-K	50 (4948 ppm)	9	King et al., 1979
Dichloromethane	Berlin-K	125-625 (10,617-33,088 ppm)	1-2	Gocke et al., 1981

[a] Discussion of references can be found in individual chapters on the respective proposed alternatives.

are based on different cell lines and different loci. However, in one study using human lymphoblasts (Gentes Limited Partnership, 1984) and another study using Chinese hamster ovary cells (Tan and Hsie, 1981), ethylene dibromide was a more potent mutagen than ethylene dichloride.

Ethylene dibromide, ethylene dichloride, and methyl bromide have been examined for DNA adduct formation in whole mammals after inhalation exposure. Although there is DNA binding data with carbon tetrachloride, it is derived from intraperitoneal injection experiments and therefore were not used in comparison with the other compounds. Liver was the only organ in which DNA alkylations could be compared for the three chemicals (Table 3-5). As can be seen in Table 3-5, methyl bromide binds DNA to a much lesser extent than does ethylene dibromide; approximately five orders of magnitude difference were observed for the alkylations per ppm·h. Although the binding of methyl bromide to DNA was measured by adduct formation on N7 guanine and not by total adduct formation, methyl bromide has a high s value (1). Compounds with a high s value have caused almost exclusively more N-alkylations, rather than mixtures of N-alkylations and O-alkylations. Therefore, it is assumed that the extent of N-alkylation for methyl bromide approximates the amount of total alkylation. Even if this assumption is inaccurate, it is highly unlikely that five orders of magnitude difference could have accounted for this difference in measurements. Ethylene dichloride appears to bind to DNA to a greater extent than methyl bromide but to a lesser extent than ethylene dibromide. It should be cautioned, however, that the measurements for ethylene dichloride are derived from a different rodent species (rat) than those for ethylene dibromide and methyl bromide (mouse). Although there were two orders of magnitude difference in the alkylations per ppm·h, a species difference could conceivably account for this difference in the amount of binding.

TABLE 3-5. DNA ALKYLATION IN LIVER IN WHOLE RODENTS

Chemical	Species	Dose (inhalation)	Total alkylations/nucleotide	Alkylations per ppm.h $\times 10^{-6}$	Reference
Ethylene dibromide	mouse	30.5 ppm.h[a]	8×10^{-6}	0.3	Lee, 1980
Ethylene dichloride	rat	900 ppm.h	4×10^{-6}	0.004	Reitz et al., 1982
Methyl bromide	mouse	144 ppm.h	3×10^{-9}[b]	0.00002	Djalali-Behzad et al., 1981

[a] Data are derived from i.p. experiments. The ppm·h equivalent was determined on the basis of equal alkylation of liver DNA in inhalation exposure 6 days post-treatment. It has been experimentally determined for ethylene dibromide that 1 ppm.h is equivalent to an i.p. injection of approximately 0.17 mg/kg.

[b] Alkylations at N7 guanine were determined in this study. Because methyl bromide has a high s value (1); it is assumed that the number of N7 alkylations will approximate the number of total alkylations.

Given the available data, few conclusions can be drawn about DNA alkylation by methyl bromide and ethylene dichloride, except that both agents interact with DNA. Methyl bromide appears to do so to a lesser extent than does ethylene dibromide. Information from experiments that involve measurements at different time intervals to determine the stability of various DNA adducts formed by these compounds would be a valuable addition to current knowledge. If stable adducts are formed in testicular DNA, which is a target tissue for heritable risk, then the induced genetic damage could accumulate during the cellular life cycle of the gonial cells. The gonia are an important cell type relevant for human genetic risk assessment. In the case of ethylene dibromide, it is known that adducts are formed in testicular DNA. Data regarding the degree of alkylation is more useful if it includes information on the type of adducts formed and the stability of these adducts. Such information is needed for ethylene dichloride, methyl bromide, and dichloromethane in germinal tissue of intact mammals.

3.3. RECOMMENDATIONS

The five proposed alternatives do not appear to be as mutagenic as ethylene dibromide. Two alternatives, carbon tetrachloride and carbon disulfide, have been primarily negative in mutagenicity testing, however it cannot be stated that they do not pose a mutagenic risk because the available information is limited and sometimes inadequate. Additional testing would be necessary for them to be classified even provisionally as nonmutagens. (Research needs for each of these chemicals are identified in Chapter 4.) It should be noted that even if these agents do not pose a mutagenic hazard, they do pose other health hazards; for example, carbon disulfide is extremely toxic and carbon tetrachloride is extremely toxic and carcinogenic in mice and rats (U.S. EPA, 1984c).

The alternative compounds that are mutagenic in several short-term gene mutation assays are ethylene dichloride, dichloromethane, and methyl bromide. It cannot be concluded that one of these agents is more mutagenic than the other because of limited data. It does appear, however, that these agents are not strong mutagens, because rather large, and often toxic, doses are required to elicit mutagenic responses. Delineation of differences in mutagenic activity among these agents will require dose-response data that are generated in the same laboratory so as to minimize technical and biological variation. The proposed alternatives are all volatile chemicals, and precautions are therefore essential to prevent excessive evaporation of test material. Several different assay systems, including mammalian systems, should be used to determine a rank order for mutagenic potency. If these experiments were coupled with molecular dosimetry, the relationship of mutation frequency could be compared to target dose rather than to exposure. If a similar rank order of potency is observed in different species, it might be reasonable to assume that a similar ranking may exist in humans. After these determinations, whole mammal germ-cell studies would have to be conducted to estimate heritable risk.

4. MUTAGENICITY OF PROPOSED ALTERNATIVES

The following sections provide a critical analysis of data pertaining to the potential mutagenicity of five proposed alternative fumigants to ethylene dibromide.

4.1. CARBON DISULFIDE

Information on the mutagenic potential of carbon disulfide has primarily been negative. Most of the testing has been conducted in bacteria. Although there are results in eucaryotic tests, the data are limited; studies have been performed by two different laboratories in Drosophila for gene mutations and in whole mammals for cytogenetic analysis. These results are discussed below and summarized in Table 4-1a.

4.1.1. Gene Mutation Studies

4.1.1.1. Bacteria--Carbon disulfide has been found to be negative in mutagenicity tests in *Salmonella typhimurium* and in *Escherichia coli*. In Salmonella, various protocols were used: preincubation, liquid suspension, fluctuation, and desiccator assays, and host-mediated assay.

Haworth et al. (1983) evaluated 250 chemicals, including carbon disulfide (MC/B, technical purity), in the Salmonella/microsome assay using a preincubation protocol. With this procedure, the liver activation system, bacteria, and test chemical are mixed and incubated for 20 minutes at 37°C. Melted top agar is then added, and the mixture is poured into petri plates and incubated for 48 hours at 37°C. Two types of S9 mix were employed: Aroclor 1254-induced rat liver and Aroclor 1254-induced hamster liver. Carbon disulfide, dissolved in DMSO, was examined at five concentrations by two different laboratories. SRI-International used concentrations of 0, 33.3, 100, 333.3, 1000, and 3333.3 ug/plate. Microbiological Associates used concentrations of 0, 20.7,

TABLE 4-1a. MUTAGENICITY TESTS ON CARBON DISULFIDE

Test system	Reported Result by Authors	Comment	Reference
A. Gene mutation studies: Bacteria			
Salmonella typhimurium (preincubation assay, liquid suspension test, fluctuation test, desiccator procedure)	-	with/without S9 mix	Haworth et al., 1983 Hedenstedt et al., 1979 Donner et al., 1981
Escherichia coli WP2 (liquid suspension, fluctuation test)	-	with/without S9 mix	Donner et al., 1981
Host-mediated assay (using Salmonella TA98 as indicator and CD-1 mice as host)	-	cannot rule out mutagenesis by base-pair substitution, suggestive increase in male-hosted bacteria	Belliles et al., 1980
Gene mutation studies: Eucaryotes			
Drosophila sex-linked recessive lethal test	-	sample size not sufficient to rule out a doubling in the lethal frequency relative to the control value	Donner et al., 1981 Belliles et al., 1980
B. Cytogenetic Effects: Whole mammals			
Rat bone marrow cells and embryonic cells	+	suggestive evidence for polyploidy and chromosome fragments at 0.1 LD_{50}	Barilyak and Vasil'eva, 1974 Vasil'eva, 1982
Rat bone marrow cells	-	dosages may have been too low	Belliles et al., 1980
Rat dominant lethal test	-	dosages may have been too low	Belliles et al., 1980
C. Other Evidence Indicative of DNA Damage:			
Unscheduled DNA synthesis in WI-38 human fibroblasts	-	a toxic dose was not examined and positive control values were weak	Belliles et al., 1980

69, 207, 690, and 2070 ug/plate. In establishing these doses, the test agent was checked for toxicity in TA100 and the highest dose tested exhibited some degree of toxicity (i.e., thinning of bacterial lawn). Both laboratories used tester strains TA98, TA100, TA1535, and TA1537 and repeated experiments at least twice. Carbon disulfide was not mutagenic either in the presence or absence of S9 mix. Although carbon disulfide is a volatile compound, it is dense (vapor density = 2.67; air = 1) and excessive evaporation would not be expected to occur with this test procotol.

Hedenstedt et al. (1979) found carbon disulfide (purity not reported, dissolved in acetone) negative in Salmonella TA100 using a desiccator procedure and a liquid suspension test. In the desiccator procedure, the bacteria were incubated for 48 hours at 37°C with 8400 ppm gaseous carbon disulfide with or without Aroclor-induced rat liver S9 mix. In the liquid suspension assay, 0, 0.63, 1.23, and 3.15 ug of carbon disulfide per ml of media were incubated in a sealed glass tube at 37°C for 1 hour. Insufficient information about procedure is given to evaluate the liquid suspension results. In addition, no concurrent positive controls were reported for this study.

Donner et al. (1981) reported carbon disulfide (source and purity not reported, dissolved in DMSO) as nonmutagenic in bacterial fluctuation tests. *Salmonella typhimurium* strains TA98 and TA100, and *Escherichia coli* WP2 *uvrA* were used as indicator organisms. Assays were performed with or without S9 mix (rat liver; inducer not reported). The concentrations used were 300, 600, and 1000 uM in Salmonella and 20, 100, 300, and 600 uM in *E. coli*. It should be noted that no positive control data were reported; thus, it is uncertain if the test system or S9 was functioning properly.

Beliles et al. (1980) used a host-mediated assay to evaluate the effects of carbon disulfide (86.97% purity: technical reagent, A.C.S.) in the presence of whole mammal metabolism. In this study, Salmonella TA98 was used as the indicator organism, and male and female mice (strain CD-1) were the hosts. The animals were exposed by inhalation 7 hours per day for 5 days to either 20 or 40 ppm carbon disulfide. Bacteria were injected intraperitoneally into the mice after the last exposure. The bacteria were retrieved from the mice 3 hours later. At the 40-ppm-dose level, small increases in the number of revertants per plate were observed for bacteria hosted in males (approximately twofold to threefold increases above the negative control) but not for those exposed in females. It is unusual that this effect was observed only in male host-mediated bacteria because there is no information that indicates a difference in response between sexes. Furthermore, a low survival was found in the male-hosted group and thus the positive result could be spurious because of clumping or differential cell growth. In addition, replicates and repeat tests were not conducted. Therefore, the positive finding in male host-mediated bacteria is considered inconclusive.

Carbon disulfide is reported to inhibit the mutagenic activity of 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) in a host-mediated assay. Moriya et al. (1979) gave male ICR mice oral doses (up to 0.5 mmol/kg body weight) of carbon disulfide (Wako Pure Chemical Industries Ltd., Tokyo, purity not reported) diluted in corn oil 2 hours before intraperitoneal injection of S. typhimurium hisG46 and the subcutaneous injection of DMH (1 mmol/kg) or AOM (0.5 mmol/kg). Three hours after the treatments, the bacteria were removed and the number of revertants and survivors were observed. Carbon disulfide exerted a dose-dependent inhibitory effect on the mutagenicity of DMH and AOM. The metabolic pathway of DMH in vivo is thought to be as follows: DMH -->

azomethane --> azoxymethane --> methylazoxymethanol. It is proposed that carbon disulfide exerts its inhibitory effects on the mutagenic activity of DMH by inhibiting the N-oxidation in vivo of azomethane to azoxymethane and also inhibiting the hydroxylation of azoxymethane to methylazoxymethanol. Carbon disulfide has been reported to inhibit the carcinogenicity of DMH (Wattenberg and Fiala, 1978).

4.1.1.2. Drosophila--Donner et al. (1981) evaluated carbon disulfide in the Drosophila sex-linked recessive lethal test. Negative results were obtained (Table 4-1b). In this experiment, 1-day-old males (w/Y) were starved for 3 hours prior to feeding carbon disulfide added to Tween 80 and mixed in 5% sucrose. Flies were allowed to feed on concentrations of 200, 500, 650, 800, and 1000 ppm (3000 mg/m^3) for 24 hours. Concentrations up to 650 ppm were nonlethal; 80% of the males died at 800 ppm and all males died at 1000 ppm. After exposure, 30 males per concentration were each mated with three females of the strain Y Basc mal. A 3 x 3 day brooding technique was used, and females were allowed to lay eggs for 6 days. The lethal frequency in the control Tween 80 group was 0.09%. Lethal frequencies in the treated were not significantly increased: 0.08% lethals at 200, 500, and 650 ppm, and 0.12% lethals at 800 ppm. The results from the different broods were pooled. Because chemicals can be germ-cell stage dependent, these data should have been reported separately, and the authors' statement that there were no differences among the broods therefore cannot be interpreted independently. The sample size in each brood in this study only rules out the possibility of a relatively strong mutagenic effect (only 4.4 to fivefold increases relative to the control value would have been detected based on the normal test and Kastenbaum-Bowman test; Margolin et al., 1983, and Kastenbaum and Bowman, 1970).

TABLE 4-1b. RESULTS OF SEX-LINKED RECESSIVE LETHAL TEST IN DROSOPHILA MELANOGASTER TREATED WITH CARBON DISULFIDE (CS_2)

Concentration of CS_2 (ppm)	Number of X-chromosomes tested	Number of recessive lethals	Frequency (%)
0 (Tween 80)	2343	2	0.09
200	2412	2	0.08
500	2394	2	0.08
650	2576	2	0.08
800	2483	3	0.12

Taken from Donner et al., 1981.

Beliles et al. (1980) also reported negative results in the Drosophila sex-linked recessive lethal test after carbon disulfide exposure. In this study, the Drosophila melanogaster males carried two repair-deficient mutations: mei-9^{a} (excision repair) and mei-41^{5} (postreplication repair). One-day-old males were starved 10 hours prior to exposure to 20 ppm (60 mg/m^3) and 40 ppm (125 mg/m^3) of carbon disulfide for 7 hours by inhalation. The percent of survivors at these concentrations was not given; these doses are not high and may be nonlethal. Four broods were examined to evaluate effects on spermatozoa, spermatids, spermatocytes, and spermatogonia (2-3-3-2 day mating scheme). Although a high number of lethals was observed in brood I at the 20 ppm dose, the majority of these lethals, however, resulted from a cluster in a single male (Table 4-1c). The cluster is likely to be spontaneous in origin rather than the result of treatment and there is no dose response. Although the authors interpreted these data as negative, they should be considered inconclusive because of the low dosages employed. Moreover, even the largest sample size (i.e., summing tests from broods 1 and 2) would have been sufficient only to detect a 3.4 to fourfold increase based on the normal test (Margolin et al., 1983) or a 4 to fivefold increase based on the Kastenbaum-Bowman test (Kastenbaum and Bowman, 1970), relative to the control value.

4.1.2. Cytogenetic Studies

Two studies on cytogenetic damage induced by carbon disulfide in rat bone marrow and embryonic cells were reported in the Russian literature (Barilyak and Vasil'eva, 1974; Vasil'eva, 1982). In one study (Barilyak and Vasil'eva, 1974), "unpedigreed" white rats were exposed to carbon disulfide and hydrogen sulfide at 10 mg/m^3. An antimitotic effect and an increase in aneuploid and polyploid cells were reported. These positive findings are difficult to evaluate because it appears that exposure was to both carbon disulfide and hydrogen

TABLE 4-1c. SUMMARY OF SEX-LINKED RECESSIVE LETHAL RESULTS FOR CARBON DISULFIDE USING REPAIR-DEFICIENT MALES OF DROSOPHILA

Compound	Concentration	Number of chromosomes scored		Number of lethals	% lethals	Number of lethals adjusted for clusters	% lethals adjusted for clusters
Negative control, filtered air	0 ppm	Brood I	1788	6 (3)[a]	0.34	3	0.17
		Brood II	1186	8 (8)[a]	0.67	0	0.0
		Brood III	918	2	0.22	2	0.23
		Brood IV	525	0	0.0	0	0.0
		Total	4417	16	0.36	5+1=6[d]	0.14
Positive control, EMS	0.015M	Brood I	185	52	28.11	Not adjusted	28.11
		Brood II	131	19	14.50		14.50
		Brood III	6	5	83.33		83.33
		Brood IV[b]					
		Total	322	76	23.91		23.91
Carbon disulfide	20 ppm	Brood I	1704	7 (5)[c]	0.41	2	0.12
		Brood II	1364	3 (1)[c]	0.22	2	0.15
		Brood III	1230	4 (2)[c]	0.33	2	0.16
		Brood IV	568	0	0.0	0	0.0
		Total	4866	14	0.29	6+1=7[d]	0.14
	40 ppm	Brood I	1347	2	0.15	No change	0.15
		Brood II	1016	3	0.30		0.30
		Brood III	685	0	0.0		0.0
		Brood IV	294	1	0.34		0.34
		Total	3342	6	0.18		0.18

[a] Lethals occurring in one male and subtracted from total; probably due to a pre-existing lethal

[b] All males died before mating.

[c] Lethals occurring in one male and subtracted from total; cluster event not attributed to treatment because they occurred in postmeiotic cells.

[d] Calculations derived as follows: Number of lethals minus lethals in cluster male plus one cluster male = total adjusted lethals.

SOURCE: Beliles et al., 1980

sulfide. In the other study (Vasil'eva, 1982), carbon disulfide (source and purity not reported) was evaluated at 1/10 and 1/100 of the LD_{50} (actual doses were not reported) in bone marrow and embryonic cells of the rat (Wistar strain). A solution of carbon disulfide in sunflower seed oil was given intragastrically to adult females over a period of 15 days. It is not clear if the administration was daily. Bone marrow cells were examined after treatment. For examination of embryonic cells, carbon disulfide was administered to female rats from the 10th to the 13th day of pregnancy. A significant increase in polyploid cells ($P < 0.01$, assuming binomial distribution) was found at 1/10 of the LD_{50}. In bone marrow, there were 0.5% polyploid cells ± 0.2 S.D. in the controls as compared to 1.6 ± 0.4 S.D. in treated animals. In embryonic cells, there were 0.7% ± 0.4 S.D. in the controls compared to 1.8% ± 0.6 S.D. in the treated group. The type of polyploidy was not defined, and it is possible that the effect may have resulted from endoreduplication or selection of a specific subpopulation of polypoid cells in the bone marrow cells. Information regarding the protocols used is insufficiently reported.

Beliles et al. (1980) tested carbon disulfide for its ability to cause chromosomal aberrations in rat bone marrow cells. Adult male and female rats [CRL:COBS CD (SD) BR] were exposed to 0, 20, or 40 ppm carbon disulfide by inhalation both acutely and subacutely. Acute exposure consisted of a single 7-hour session in the exposure chamber. Subacute exposures consisted of 5 daily exposures of 7 hours. Bone marrow cells were then collected, slides were prepared and coded, and 50 cells per animal were scored. There was no increase in the number of aberrations or aneuploidy in either study; however, these studies are not considered adequate tests of the potential of carbon disulfide to cause chromosome damage or to cause aneuploidy. The positive control, triethylenemelamine

(TEM), was given by i.p. injection while carbon disulfide was administered by inhalation. The concentrations tested in these studies are based on the threshold limit value in air (20 ppm) and not based on the LD_{50}. The lowest acute concentration in air reported to cause death in mammals is 2000 ppm for 5 minutes. Thus, the doses may have been too low to detect clastogenic activity.

Beliles et al. (1980) also assessed the ability of carbon disulfide in air to cause dominant lethal effects in rats [CRL: COBS CD (SD) BR]. The precise nature of damage causing dominant lethal effects is not known, but there is a good correlation between chromosome breakage in germ cells and dominant lethal effects (Matter and Jaeger, 1975). Males (10 animals/dose) given doses of 0, 20, or 40 ppm carbon disulfide by inhalation 7 hours per day for 5 days were mated to unexposed females during a 7-week period. The authors indicated that a dose-related increase in dead implants per total implant was observed at week 7 but the increase at each dose level was not significantly different from the controls. These data were not reported. The doses used in this test may not have approximated the maximum tolerated dose, and even if the maximum tolerated dose was approached, the dominant lethal test is not a particularly sensitive test for detecting mutagens in view of the high spontaneous frequency of fetal wastage (Russell and Matter, 1980).

4.3.1. Other Studies Indicative of DNA Damage

Unscheduled DNA synthesis (UDS) is a measure of the repair of DNA lesions and is indicative of DNA damage. Beliles et al. (1980) assessed the ability of carbon disulfide to induce UDS in human (WI-38) lung fibroblasts. UDS is detected by measuring the incorporation of tritiated thymidine (^{3}H-TdR) into DNA in the absence of semiconservative replication. Liquid scintillation counting was used to measure the amount of ^{3}H-TdR incorporation. When cells were exposed to carbon disulfide at doses ranging from 0.1 to 5.0 ul/ml, no increases in UDS over control values were observed either with or without metabolic activation (Aroclor 1254-rat liver S9 mix). The positive controls in this study responded only weakly: N-methyl-N'-nitro-N-nitrosoguanidine elicited only a 1.8-fold increase over background UDS; and the control requiring metabolic activation, benzo[a]pyrene, gave only a 1.16-fold increase, which is an equivocal response. Moreover, the highest concentration of carbon disulfide tested did not inhibit the incorporation of ^{3}H-TdR; higher concentrations should have been tested to reach a toxic level. Replicate plates were not used in this study. Because of these inadequacies, the negative results reported for carbon disulfide must be considered inconclusive.

4.1.4. Gonadal Studies

If a mutagen reached germinal tissue, it would have the potential to cause damage that could contribute to the burden of genetic disease.

Lancranjan (1972) and Lancranjan and coworkers (1969) reported a reduction in sperm counts and altered sperm morphology in workers with occupational exposure to carbon disulfide. In one study, germinal effects were observed in 140 male workers (average age 30 years) suffering from chronic carbon disulfide poisoning. Semen samples from these workers showed a high incidence of asthenospermia, hypospermia, and teratospermia compared with 50 matched controls.

Meyer (1981) studied semen quality of 86 exposed workers for at least a year. There were three defined exposure levels: high exposure (>10 ppm) in 18 workers, moderate exposure (2-10 ppm) in 27 workers, and low exposure (>2 ppm) in 22 workers. A fourth group with uncertain exposure included 19 workers. No statistically significant effects on sperm counts or sperm morphology were found in the workers compared with a control group (about 89 controls). The differences were nonsignificant whether the control group was compared with the exposed group as a whole or with each of the subgroup exposures. Complications in this study are that the length of employment differed among the subgroups (workers had to be employed at least 12 months to participate) and some exposures may have been too brief to cause detectable changes. It is not clear whether exposure was constant or intermittent, and the basis for conclusions would have been stronger if more workers had participated.

Carbon disulfide has been shown to cause semen changes in experimental mammals at high doses. Tepe and Zenick (1984) studied male rats (adult Long Evans hooded) exposed to 0, 350, or 600 ppm carbon disulfide for 10 weeks (5 hours/day, 5 days/week). Animals exposed to the high dose (600 ppm) had slightly lower epididymal sperm counts and significantly reduced plasma testosterone levels.

Beliles et al. (1980) examined carbon disulfide for its ability to cause altered sperm morphology in treated rats [CRL: COBS CD (SD) BR] and mice (CD-1). After treatment with 0, 20, and 40 ppm by inhalation 7 hours per day for 5 consecutive days. Groups of four animals were killed at the end of 1, 4, and 10 weeks to examine effects on various germ-cell stages. Sperm were collected from the cauda epididymis, and at least 500 cells were examined. Negative results were reported both in rats and mice. It should be noted, however, that the positive control TEM proved essentially inactive in the rat assay, except for a small increase during week 10; this increase indicates the lack of sensitivity of the test. In the mouse, TEM showed an effect at week 4; but carbon disulfide and the negative control were also slightly elevated during week 4. The results of this study are regarded as inconclusive.

Although carbon disulfide affects the male reproductive system in certain tests, it is not certain that these effects result from carbon disulfide reaching the germinal tissue; the effects on male reproduction can also result from altered hormone levels and/or general toxicity. A more in-depth analysis of the potential reproductive effects of carbon disulfide can be found in the REAG report entitled, "Assessment of the Reproductive and Developmental Toxicity Potential of Carbon Disulfide" (prepared by Dr. Carole A. Kimmel for OPP, November 14, 1984).

4.1.5. Summary and Conclusions

There is no evidence that carbon disulfide is mutagenic in bacteria. Two studies have been performed in Drosophila, and negative results were reported. Only relatively strong effects, however, would have been detected in these studies. There is suggestive evidence for the ability of carbon disulfide to cause polyploidy and chromosome fragments in rat bone marrow cells and embryonic cells, but this evidence is derived from one study which is inadequate in several respects. In another study, negative results were found in rat bone marrow cells; but the dosage may have been too low for the detection of chromosome abnormalities. Although the results of carbon disulfide have been predominantly negative, the possibility of weak mutagenicity cannot be ruled out. The inability to draw a firm conclusion regarding the mutagenic potential of carbon disulfide emphasizes the need for additional testing. It is particularly important that studies be designed to permit the detection of weak mutagenic activity (e.g., sufficient sample sizes and concentration range). The focus should be on the testing of eucaryotes rather than bacteria, and tests for chromosome nondisjunction should be included.

4.2. CARBON TETRACHLORIDE

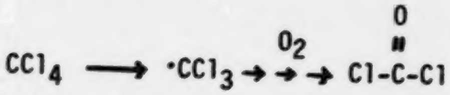
The mutagenic potential of carbon tetrachloride (CCl_4) has been assessed by evaluating the results of seven bacterial studies, one yeast study, one in vitro mammalian chromosome damage study, and three in vivo DNA damage studies in rodents. The majority of these studies were negative. Information relating to the metabolism of CCl_4 and to covalent binding of the metabolites to cellular macromolecules (including DNA) precedes the sections assessing the genotoxicity of CCl_4. This was done to set the stage for the discussion of the largely negative results obtained in the mutagenicity studies and for the suggestion that CCl_4 may be a weak mutagen. Recommendations for additional testing are presented.

4.2.1. Metabolism and Covalent Binding to Macromolecules

The evidence described in this section suggests that CCl_4 is metabolized in the liver to highly reactive intermediates (the trichloromethyl free radical and phosgene). The evidence also indicates that metabolically activated CCl_4 covalently binds to protein, lipid, and DNA, suggesting that CCl_4 may have genotoxic potential.

4.2.1.1. Metabolism--CCl_4 is metabolized in the liver endoplasmic reticulum by the cytochrome P-450 component of the mixed-function oxidase system (Reynolds and Moslen, 1980). The available evidence indicates that metabolism of CCl_4 results in the generation of the trichloromethyl free radical $\cdot CCl_3$ (Reynolds and Moslen, 1980; Trudell et al., 1982) and phosgene (Shah et al., 1979; Kubic and Anders, 1980; Pohl et al., 1981). Because of their high reactivity, these two substances are the most likely metabolites to interact with tissue macromolecules. By using human cytochrome P-450 reconstituted in phospholipid vesicles, Trudell et al. (1982) have demonstrated that $\cdot CCl_3$ is the major product of the reductive metabolism of CCl_4 as determined by mass spectral identification of the adducts formed between $\cdot CCl_3$ and the phospholipid dioleoyl phosphatidylcholine.

Phosgene is produced from further metabolism of $\cdot CCl_3$ (Shah et al., 1979).

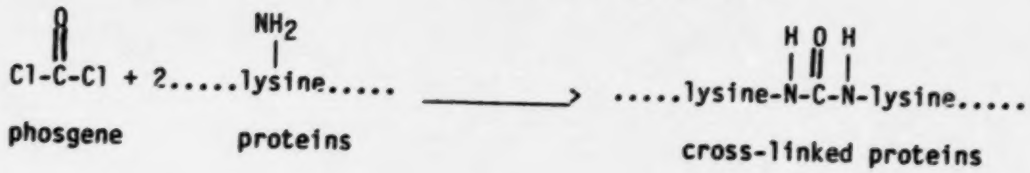


$$CCl_4 \longrightarrow \cdot CCl_3 \rightarrow \overset{O_2}{\rightarrow} \rightarrow Cl\text{-}\overset{\overset{O}{\|}}{C}\text{-}Cl$$

Pohl et al. (1981) measured the amount of phosgene (as diglutathionyl dithiocarbonate) produced in the aerobic metabolism of CCl_4, $CHCl_3$, and $CBrCl_3$ by liver microsomes from phenobarbital-treated rats plus cofactors. The results indicate that phosgene production from CCl_4 is only 4% of that produced from $CHCl_3$. Thus, the level of phosgene production from aerobic metabolism of CCl_4 is relatively small. Metabolites of CCl_4 are so reactive that they bind to and inactivate the cytochrome P-450 enzymes that were responsible for their generation (suicide mechanism) (Vainio et al., 1976; Sipes et al., 1977; De Groot and Haas, 1980; Cooper and Witmer, 1982).

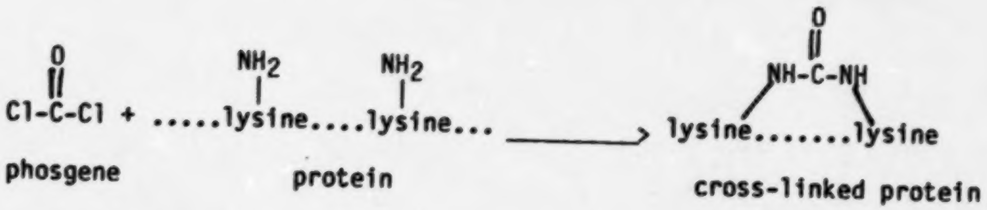
4.2.1.2. Covalent Binding--Metabolically activated CCl_4 has been found to bind to lipid and protein both in vivo (Rocchi et al., 1973; Diaz Gomez and Castro, 1980a, b) and in vitro (Rocchi et al., 1973; Uehleke et al., 1977). The amount of label bound was determined after washing and extraction, indicating that the binding was covalent. Uehleke et al. (1977) measured covalent binding of 1 mM $^{14}CCl_4$ (0.25 Ci/mol) to microsomal protein and lipid in liver microsome suspensions (5 mg protein per ml plus cofactors) from phenobarbital-treated rabbits. About 10% of the ^{14}C label was covalently bound to endoplasmic reticulum protein, and greater than 30% was bound to microsomal lipid. Extramicrosomal binding was evaluated by the addition of 5 mg of bovine serum albumin per ml to the CCl_4/microsome mixture. The binding of metabolically activated $^{14}CCl_4$ to the bovine serum albumin (1.4 nmol/mg in 60 min) was about 1.5% of that bound to microsomal protein (20.0 nmol/mg) plus lipid (76.0 nmol/mg). Thus, it appears that binding of metabolically activated $^{14}CCl_4$ to extramicrosomal macromolecules is small compared with binding to microsomal constituents.

Evidence that the CCl_4 metabolite, phosgene, reacts with proteins was obtained by Cessi et al. (1966) when they measured the in vivo binding of CCl_4 to rat liver proteins and compared it with the in vitro acylation of poly-L-lysine and serum albumin by phosgene. Similar reaction products were obtained in both systems, suggesting that phosgene reacts with the ε-amino groups of lysine in proteins, leading to cross-linked carbonyl derivatives:



$$\text{Cl-}\overset{\overset{O}{\|}}{\text{C}}\text{-Cl} + 2\text{.....}\overset{\overset{NH_2}{|}}{\text{lysine}}\text{.....} \longrightarrow \text{.....lysine-}\overset{\overset{H}{|}}{\text{N}}\text{-}\overset{\overset{O}{\|}}{\text{C}}\text{-}\overset{\overset{H}{|}}{\text{N}}\text{-lysine.....}$$

or



$$\text{Cl-}\overset{\overset{O}{\|}}{\text{C}}\text{-Cl} + \text{.....}\overset{\overset{NH_2}{|}}{\text{lysine}}\text{....}\overset{\overset{NH_2}{|}}{\text{lysine}}\text{...} \longrightarrow \overset{\text{NH-}\overset{\overset{O}{\|}}{\text{C}}\text{-NH}}{\text{lysine.......lysine}}$$

Such cross-linked proteins would exhibit impaired biological activity. It is also possible that similar cross-linking reactions of phosgene can occur with amino groups in DNA resulting in alterations in DNA structure and function.

Binding of metabolically activated CCl_4 to DNA was found by two groups. Rocchi et al. (1973) studied the binding of CCl_4 to nucleic acids and protein. ^{14}C-Labeled CCl_4 (367 umol/kg) was injected into rats and mice, and the amount of metabolite(s) of CCl_4 that covalently bound to liver DNA, RNA, nuclear proteins, and cytoplasmic proteins was measured. Significant amounts of labeled material were found associated with rRNA, nuclear proteins, and cytoplasmic proteins in rats. When the rats were pretreated with 3-methylcholanthrene (3-MC) (5 mg, 24 hours before treatment with CCl_4), the amount of label associated with the macromolecules increased. No label was associated with DNA in the rat studies, but similar studies in mice indicated that DNA binding occurs (108 umol CCl_4/mol DNA). Binding to DNA was observed only after pretreatment of the mice with 3-MC (1 mg, 24 hours before treatment with CCl_4).

In an in vitro experiment, Rocchi et al. (1973) used rat or mouse liver microsomes to activate labeled CCl_4 in the presence of calf thymus DNA. Pretreatment of animals with 3-MC enhanced the amount of label associated with DNA. Furthermore, pH 5 enzyme preparations containing activating enzymes (Keller and Zamecnik, 1956) also were found to increase the amount of label bound to DNA. It therefore appears that metabolites of CCl_4 can interact with DNA; for optimal binding, microsomal enzymes required that activation with 3-MC and the binding assay be carried out in the presence of pH 5 enzymes.

Diaz Gomez and Castro (1980a) also measured binding of metabolically activated CCl_4 to cellular macromolecules. ^{14}C from $^{14}CCl_4$ (specific activity, 27 Ci/mol) irreversibly bound in vivo to liver nuclear DNA, protein, and lipids in strain A/J mice and Sprague-Dawley male rats. Mouse and rat liver DNA isolated from animals given $^{14}CCl_4$ 16 hours before they were killed exhibited a small but significant labeling (mice, 0.72 pmol/mg; rats, 0.52 pmol/mg). The count from the assay carried out in the presence of unlabeled DNA was subtracted from the experimental counts before binding was calculated. In contrast to the results of Rocchi et al. (1973), induction of liver enzymes by 3-MC was not required for binding of ^{14}C from $^{14}CCl_4$ to DNA. Although the purified DNA samples contained 0.2% protein, contamination by protein at this low level could not account for all the covalent binding measured in the DNA sample.

In vitro binding of metabolically activated CCl_4 to isolated mouse liver DNA (1.81 pmol/mg) was observed by Diaz Gomez and Castro (1980a) in anaerobic incubation mixtures containing microsomes and NADPH. It was also found that $\cdot CCl_3$, produced by reaction of CCl_4 with benzoyl peroxide, interacted with DNA (826 pmol/mg). This result suggests that $\cdot CCl_3$ may be the chemical species involved in the binding to DNA.

In addition to observing that metabolites of CCl_4 bind to DNA, Diaz Gomez and Castro (1980a) observed binding of metabolically activated CCl_4 to rat liver nuclear protein and lipid in vivo. The label bound to nuclear protein was 47.7 pmol/mg and that bound to nuclear lipid was 113.5 pmol/mg. Diaz Gomez and Castro suggested that binding to nuclear lipids may be a significant finding regarding the potential carcinogenicity of CCl_4, because nuclear lipid is derived from the nuclear membrane, which contains the cytochrome P-450 necessary for the activation of CCl_4 to the reactive metabolites $\cdot CCl_3$ and phosgene. Since these metabolites are highly unstable and not likely to exist long enough to travel from the endoplasmic reticulum to the nucleus, activation by nuclear membrane P-450 enzymes is more likely to allow the metabolites to react with DNA than is activation in the endoplasmic reticulum.

Diaz Gomez and Castro (1980b) determined the potential of purified rat liver nuclei to activate CCl_4 by measuring covalent binding of nuclear-activated CCl_4 to nuclear protein and lipid. Binding to DNA was not measured. The results were compared with those results obtained from similar incubation mixtures containing microsomes instead of purified nuclei. The incubation mixtures containing either nuclei (1.3 mg protein/ml) or microsomes (1.56 mg protein/ml) were incubated for 30 minutes in 37.6 nM $^{14}CCl_4$ (6.94 Ci/mol) and an NADPH generating system in an O_2-free N_2 atmosphere. The extent of binding to proteins in the nuclear preparations was 43.5% of that observed for microsomes (nuclear suspensions, 21.9 pmol/mg; microsomes, 50.3 pmol/mg). Binding to nuclear lipids was 77.3% of that observed for microsomes (nuclear suspension, 147 pmol/mg; microsomes, 190 pmol/mg). Isolated nuclei were less efficient than microsomes in metabolizing CCl_4, but the results were within the same order of magnitude. This study indicates that metabolism of CCl_4 to reactive intermediates can occur in nuclear membranes and suggests that the in vivo binding observed in the previous study (Diaz Gomez and Castro, 1980a) may have been due to nuclear rather than microsomal activation of CCl_4. It should be mentioned, however, that the nuclear preparations were contaminated with trace amounts of endoplasmic reticulum, which may have contributed to the nuclear activation observed.

Diaz Gomez and Castro (1981) have published preliminary evidence that $\cdot CCl_3$, chemically generated from the benzoyl peroxide-catalyzed decomposition of CCl_4, reacts with guanine and adenine and to a lesser extent with cytosine and thymine. This result suggests that $\cdot CCl_3$ may bind to DNA in vivo by interaction with the purine and pyrimidine acid bases.

In summary, it has been shown that CCl_4 is metabolized to the reactive intermediates $\cdot CCl_3$ and phosgene and that metabolically activated CCl_4 binds to DNA, protein, and lipid. These results suggest that CCl_4 has genotoxic potential. The negative results in six of the seven bacterial mutagenicity studies described in section 4.2.2.1. may be due to inadequate metabolic activation in the test systems or to scavenging by protein or lipid of any very reactive metabolic intermediates (e.g., $\cdot CCl_3$ and phosgene) formed under conditions of exogenous activation.

4.2.2. Gene Mutations Studies

4.2.2.1. Bacteria--Studies to determine the mutagenic activity of CCl_4 in the Salmonella typhimurium revertant system have been negative. A review by McCann et al. (1975) stated that an assay using Aroclor-induced S9 activation and strains TA100 and TA1535 was negative, but details of the procedure were not given. In another review article without data, Fishbein (1976) reported that CCl_4 was not mutagenic when assayed in a spot test with strain TA1950.

Uehleke et al. (1977) tested the mutagenicity of CCl_4 in suspension assays with S. typhimurium strains TA1535 and TA1538. No mutagenic activity was detected. About 6-9 x 10^8 bacteria were incubated for 1 hour under N_2 in tightly closed test tubes with 8 mM CCl_4 and microsomes (5 mg protein) plus cofactors. The mutation frequencies (His^+ colony forming units/10^8 his^- colony forming units) were less than 10 for both strains, and the spontaneous mutation frequencies were 3.9 ± 3.7 for strain TA1535 and 4.4 ± 3.5 for strain TA1538. At this concentration of CCl_4, survival of the bacteria was at least 90%. This negative result for CCl_4 is questionable because concentrations that result in less than 90% survival should have been tested. Dimethylnitrosamine, cyclophosphamide, 3-methylcholanthrene, and benzo[a]pyrene were the positive controls used in this study. Although these chemicals were mutagenic in the presence of the S9 activation system, they are not ideal controls for CCl_4 because they are not halogenated alkanes and are not metabolized like CCl_4.

Uehleke et al. (1977) suggested that any reactive species generated by the microsomes may not have reached the bacteria, resulting in the negative test. It is not clear from the description whether rat, mouse, or rabbit microsomes were used in the mutagenicity studies. It is clearly stated that rabbit microsomes were used for the binding studies described previously. If mouse or rat microsomes rather than rabbit microsomes were used for the mutagenicity experiments, it cannot be assumed that CCl_4 was sufficiently activated, since activation sufficient for binding of $^{14}CCl_4$ to macromolecules was shown in this paper only with rabbit microsomes. Another deficiency in this study is that the Salmonella strains TA98 and TA100 were not used. These strains contain an R factor plasmid that increases the sensitivity of the tester strains to certain mutagens. Because of these deficiencies, the negative mutagenicity results in this paper should be regarded as inconclusive.

The mutagenicity of CCl_4 was also tested in a study on the mutagenic potential of chemicals in drinking water (Simmon et al., 1977). No mutagenic activity was detected with CCl_4. The authors tested 71 of the 300 chemicals that had been identified in public water supplies. CCl_4 was tested in a desiccator to assess mutagenicity due to vapor exposure and to avoid excessive loss of CCl_4 to the atmosphere. The desiccator contained a magnetic stirrer that acted as a fan to aid in evaporation of the measured amount of CCl_4 and to maintain an even distribution of the vapors. Plates were exposed to the vapors for 7 to 10 hours and then removed from the desiccators, covered, and incubated approximately 40 hours before scoring. Mutagenic activity was not observed and no information on toxicity was provided.

The study by Simmon et al. (1977), although lacking some specific details of the CCl_4 assay, clearly identifies certain trihalomethanes ($CHBr_3$, $CHBr_2Cl$, $CHBrCl_2$) as mutagens in the vapor assay in desiccators. Methyl bromide, methyl chloride, methyl iodide, and methylene chloride were also found to be mutagenic in the desiccator assay. However, these seven halogenated compounds did not require metabolic activation to exhibit mutagenic activity. It may be that CCl_4 itself is not mutagenic and the rat liver S9 does not effectively metabolize

CCl_4 to a mutagen, even though the mutagenicity of three of the chemicals tested [bis(2-chloroisopropyl)ether, vinyl chloride, and vinylidene chloride] required or was enhanced by the rat liver S9 mix. It may also be that a reactive intermediate was formed but was too short-lived to be detected in a test system that uses exogenous metabolic activation.

Another negative result for the mutagenicity of CCl_4 was obtained in a variation of the Salmonella/microsome assay in which the escape of volatile compounds was prevented by the use of a specially designed, closed, inert incubation system (Barber et al., 1981). Seven of the 10 halogenated alkane solvents tested gave positive mutagenicity results when the assays were carried out in the closed incubation system. Under standard conditions (in which volatilization was not prevented), only 2 of the 10 solvents gave a positive result. Thus, the closed incubation system allowed for the detection of five more mutagens than could be detected under standard conditions. CCl_4 was one of the three solvents that gave a negative result in both the standard and closed incubation systems. The investigators indicated that CCl_4 was tested at concentrations high enough to produce observable toxicity, determined by the absence of background lawn. The Salmonella strains used were TA1535, TA1537, TA1538, TA98, and TA100. Levels of CCl_4 tested were 4.7, 5.7, 10.2, 12.3, and 18.4 umol per plate, and no dose-related response was observed. The seven solvents that were mutagenic in this closed system did not require metabolic activation by S9 mix, but the S9 mix did activate the positive control 2-aminoanthracene. It is also possible that the S9 mix used, although adequate for activation of the control 2-aminoanthracene, was not adequate to metabolize CCl_4. It is also possible that active metabolites, if formed, reacted with microsomal components or bacterial membrane macromolecules before reaching the bacterial DNA.

Cooper and Witmer (1982) reported in an abstract that exposure of Salmonella strain TA100 to CCl_4 for 20 minutes in a 1-ml liquid suspension at low oxygen tension (3-ml Vacutainer tubes) before preparation of plates resulted in a twofold increase in revertants above background (background, 72 ± 9 revertants; 5 umol CCl_4, 141 ± 20 revertants). Evidence for a dose-response relationship was not reported. The weak mutagenic result was observed in two separate experiments and only when fresh rabbit liver S9 was used as the activation system. It is unlikely that the rabbit liver S9 alone was responsible for the mutagenic activity observed, because plate assays with rabbit S9 exhibited no mutagenic activity. The CCl_4 was spectrophotometric grade (Spectrar) purchased from Mallinckrodt (Dr. Charlotte Witmer, personal communication). CCl_4 was toxic in this suspension assay; 2 umol/ml caused 80% toxicity with microsomal activation and 20% without activation. Addition of 0.1 mM EDTA to inhibit microsomal lipid peroxidation decreased bacterial toxicity. The investigators concluded that the suspension assay in Vacutainer tubes may be a suitable system for testing volatile compounds that undergo reductive metabolism. The marginal response in this assay suggests that CCl_4 may be mutagenic in the Salmonella assay, but only under certain assay conditions (low oxygen tension, exposure of bacteria in suspension in the presence of the EDTA, and use of fresh rabbit liver S9). In view of the very weak response and lack of evidence for dose-dependence, however, the study should be regarded as inconclusive.

In summary, the results of bacterial tests of CCl_4 are predominantly negative but inconclusive. False negative results could have been obtained due to a number of factors, including:

1. The activation systems used may have been inadequate for metabolism of CCl_4.

2. $\cdot CCl_3$ and phosgene, the primary reactive metabolites of CCl_4, are unstable and highly reactive. Because exogenous activation systems were used in many of these studies, any $\cdot CCl_3$ or phosgene generated may have been scavenged by microsomal protein or lipid before reaching the DNA.

3. Adequate exposure to CCl_4 may not have occurred if appropriate precautions were not taken to prevent the evaporation of CCl_4.

4.2.2.2. Yeast--Callen et al. (1980) studied the genetic activity of CCl_4 in strain D7 of *Saccharomyces cerevisiae*, which contains an endogenous cytochrome P-450 dependent monooxygenase system. By using yeast, Callen and his coworkers eliminated the need for the exogenous type of metabolic activation system used in the bacterial studies. Three different genetic endpoints can be examined in strain D7: gene conversion at the *trp5* locus, mitotic crossing over at the *ade2* locus, and reversion at the *ilv1* locus. The effect of CCl_4 (Mallinckrodt, 99+% pure) on these endpoints was measured by exposing cells in suspension to 3.23, 4.31, and 5.13 g of CCl_4 per liter of buffer (21 mM, 28 mM, and 34 mM, respectively), well above the solubility level of CCl_4 in water (0.8 g/L at 25°C). A dose-response relationship could not be obtained because the dose was essentially the same in all cases--the solubility level of CCl_4 in water at 37°C. Escape of CCl_4 was minimal because the incubations were carried out in screw-capped glass tubes. Although the dose is essentially constant, amounts in suspension vary. Extracellular or membrane effects may have resulted in the high toxicity observed at 5.13 g/L.

Results of Callen et al. (1980) are presented in Table 4-2. A 1-hour treatment of cells with the highest amount of CCl_4 tested (5.13 g/L) resulted in significant increases in gene conversion (31-fold) and mitotic crossing-over (25-fold). Reversion was also increased, but to a lesser extent (threefold increase). Survival was only 10% at this concentration of CCl_4. At the intermediate level (4.31 g/l) of CCl_4, much weaker effects on the three genetic endpoints were observed (two to threefold increases). Survival at this level was 77%. The data of Callen et al. (1980) suggest a positive response, but because of the solubility problem, additional studies are needed before it can be stated conclusively that CCl_4 causes genetic effects in yeast.

4.2.3. Cytogenetic Studies

Negative results have been obtained in an in vitro chromosome assay in an epithelial-type cell line derived from rat liver (Dean and Hodson-Walker, 1979). This cell line has sufficient metabolizing activity to activate various chemical mutagens and carcinogens without the need for exogenous metabolic activation. Sealed-flask cultures were treated for 22 hours with CCl_4 dissolved in growth medium at 0.005, 0.010, and 0.020 mg/l. At these low concentrations, CCl_4 did not induce chromatid or chromosomal aberrations, whereas a number of direct-acting mutagens and several compounds that require metabolic activation produced chromatid deletions, gaps, and exchanges. No other heavily chlorinated substances were tested. In addition, most of the compounds were assayed at doses several orders of magnitude higher than that of CCl_4. The doses chosen for each substance assayed were determined from cytotoxicity tests. CCl_4 was apparently toxic to rat liver cells. EDTA (0.1 mM) has been found to decrease the cytotoxicity of CCl_4 without affecting mutagenicity in bacteria (Cooper and Witmer, 1982); perhaps addition of EDTA to a mammalian in vitro chromosome assay, such as that used by Dean and Hodson-Walker (1979), would permit the use of larger concentrations of CCl_4.

4.2.4. Other Studies Indicative of DNA Damage

Mirsalis and Butterworth (1980) measured unscheduled DNA synthesis (UDS) in primary rat hepatocyte cultures following in vivo treatment of adult male Fisher 344 rats (200-250 g) with CCl_4 (certified ACS grade, Fisher Scientific

TABLE 4-2. GENETIC EFFECTS OF CARBON TETRACHLORIDE ON STRAIN D7 OF *Saccharomyces cerevisiae* FOLLOWING 1 HOUR TREATMENT AT 37°C[a]

	Concentration, g/L			
	0	3.23	4.31	5.13
Survival				
Total colonies	1454	1252	1120	152
% of control	100	86	77	10
trp5 locus (gene conversion)				
Total convertants	285	331	350	506
Convertants/10^5 survivors	2.0	2.6	3.1	61.7
ade2 locus (mitotic crossing over)				
Total twin spots	1	3	3	10
Mitotic cross-overs/10^4 survivors	1.6	5.3	5.8	40.1
Total genetically altered colonies	11	19	16	65
Total genetically altered colonies/ 10^3 survivors	1.7	3.4	3.1	33.3
ilv1 locus (gene reversion)				
Total revertants	38	41	57	11
Revertants/10^6 survivors	2.6	3.3	5.1	7.2

[a] The number of colonies in the different classes represent total counts of colonies from five plates in the case of survival, conversion, and revertant-frequency estimations. Mitotic crossing over was estimated from counts of colonies growing on a total of 30 plates, 20 plates containing medium on which all surviving cells grew, and 10 plates containing medium on which only *trp5* convertants grew.

SOURCE: Adapted from Callen et al., 1980.

Co.) by oral gavage. Control rats received corn oil by gavage. Acetylaminofluorine and dimethylnitrosamine were also tested as positive control substances. At 2 hours after treatment, the livers were perfused in situ and hepatocytes were isolated. Approximately 6 x 10^5 viable cells were seeded in culture dishes and allowed to attach to coverslips for 90 minutes. The coverslip cultures were washed and then incubated for 4 hours in medium containing 10 uCi [^{3}H]thymidine (42 Ci/mmol) per ml. The cultures were washed again and incubated in medium containing 0.5mM cold thymidine for 14-16 hours. The extent of UDS was assessed by autoradiography. Net grains per nucleus were calculated as the silver grains over the nucleus minus the highest grain count of three adjacent nuclear-sized areas over the cytoplasm. The area of the silver grains, rather than the grain number was determined so that UDS could be accurately measured in densely labeled cells in which silver grains were touching.

Cell counts from control animals had from -2 to -6 net grains per nucleus. Treatment of rats with dimethylnitrosamine (i.p.) at 10, 1, or 0.1 mg/kg yielded 36.6, 6.4, and -0.9 net grains per nucleus, respectively; dimethylnitrosamine at 10 mg/kg (p.o.) produced 22.2 net grains per nucleus. Oral doses of acetylaminofluorine at 50 and 5 mg/kg yielded counts of 14.0 and 6.4 net grains per nucleus, respectively. CCl_4 at 100 or 10 mg/kg (p.o.) yielded counts of 3.2 and 5.1 net grains per nucleus, respectively. Thus, dose-related increases in UDS were observed for the positive control dimethylnitrosamine and acetylaminofluorine, whereas CCl_4 produced no such response.

As indicated above, two doses of CCl_4 were tested: 10 mg/kg and 100 mg/kg. The oral LD_{50} for CCl_4 in rats is 2800 mg/kg. The dose at which hepatic cell toxicity would occur under the conditions used was not determined and it is not clear whether adequate doses of CCl_4 were tested. It is also unclear whether the 2-hour time period between exposure of the rats to CCl_4 and isolation of the hepatocytes was sufficient for observation of UDS. In a study by Popp et al. (1978) in which CCl_4-induced hepatocellular changes were noted, the shortest exposure period studied was 6 hours. If it were shown that the 2-hour exposure period is sufficient for activation of the CCl_4 to a reactive intermediate, for example, by demonstrating alkylation of protein by $^{14}\cdot CCl_3$, the negative results would be more convincing. The negative result may reflect the inability of CCl_4 to cause UDS or it could be a false negative result due to factors such as inadequate dose or inadequate exposure time.

Craddock and Henderson (1978) carried out an in vivo UDS study in which hepatocyte nuclei were isolated and then assayed for radioactivity by scintillation counting rather than by grain counting. This study used a CCl_4 dose of 4000 mg/kg, which is significantly larger than the oral LD_{50} (2800 mg/kg). Negative results were obtained after a 2-hour exposure, but a positive response was observed after a 17-hour exposure. The investigators suggested that this result may be attributable to secondary effects such as lysosomal damage, which may result in release of DNA degradative enzymes.

In their latest study, Mirsalis et al. (1982) used combinations of doses (up to 400 mg/kg) and exposure times (up to 48 hours) that resulted in liver toxicity. Criticisms of previous studies relating to inadequate dose and exposure have been obviated by this study. The results for CCl_4 were negative in this study as well. However, benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, and N-methyl-N'-nitro-N-nitrosoguanidine were negative in this in vivo UDS assay, whereas these chemicals tested positive in the in vitro rat hepatocyte UDS assay (Williams, 1981). This discrepancy suggests that the in vitro test may be more sensitive than the in vivo assay. CCl_4 has not been tested in the in vitro rat hepatocyte UDS assay.

In summary, the in vivo UDS results provide no evidence that CCl_4 causes DNA damage that elicits UDS. However, in vitro UDS studies are needed before a firm conclusion is reached about CCl_4 not inducing UDS.

4.2.5. Suggested Additional Testing

Suggested additional testing falls into six categories:

1. The DNA damage studies reported by Craddock and Henderson (1978), Mirsalis and Butterworth (1980), and Mirsalis et al. (1982), which provide no evidence that CCl_4 induce UDS following in vivo treatment of rats and should be corroborated by the in vitro rat hepatocyte UDS assay of Williams (1981). The in vitro test may be more sensitive than the in vivo test for weak genotoxic effects.

2. Studies using sensitive assay procedures for detecting the formation of chemical adducts in liver DNA after exposure of animals to CCl_4 are needed to confirm the low level of DNA binding observed by Diaz Gomez and Castro (1980a) and by Rocchi et al. (1973).

3. Additional data from yeast studies are needed to confirm the study of Callen et al. (1980) in the yeast system, which utilizes an endogenous activation system and is capable of assaying for point mutations, mitotic crossing over, and gene conversion.

4. Additional cytogenetic testing in mammalian systems is needed before CCl_4 can be considered to be adequately tested for chromosome damage. Because EDTA has been reported to decrease the cytotoxicity of CCl_4 in bacteria (Cooper and Witmer, 1982), in vitro mammalian liver cell cytogenetic assays should be carried out in the presence and absence of EDTA. The EDTA may allow higher levels of CCl_4 to be assayed than were used in the study by Dean and Hodson-Walker (1979). In vivo studies, such as bone marrow cytogenetic analysis or a micronucleus test, are also needed. Tests for nondisjunction should be included.

5. A thorough test is needed to corroborate (or refute) the preliminary evidence for a weak mutagenic response in Salmonella reported by Cooper and Witmer (1982). The same experimental conditions (fresh rabbit liver S9 and exposure of the bacteria in suspension to CCl_4 under reduced oxygen tension in the presence of EDTA) should be used, and several concentrations of CCl_4 should be tested to determine if a dose-response relationship exists. If the bacterial test is positive, in vitro mammalian mutagenicity studies should be carried out under the same experimental conditions. If the bacterial test is negative, the cell culture assay should be conducted by a more standard protocol with provision for the volatility of the chemical.

6. Studies on the ability of CCl_4 to reach reproductive organs and cause germ-cell mutation have not been conducted. If results from the tests for mutation in cultured cells are positive, then studies assessing heritable risk are needed. The document entitled, Proposed Guidelines for Mutagenicity Risk Assessment, published in the Federal Register in 1984, is recommended for guidance on such tests.

4.2.6. Summary and Conclusions

CCl_4 has been tested for its mutagenic potential in bacteria, yeast, and a mammalian cell line and for its DNA damaging potential in rat hepatocytes when administered in vivo.

Six of the seven point mutation studies in bacteria were negative. The remaining bacterial study (Cooper and Witmer, 1982) was a preliminary test that provided only suggestive evidence of a weak mutagenic response. In none of the negative studies was it shown that CCl_4 was activated or metabolized by the exogenous S9 activation system used. Metabolism of 2-aminoanthracene or vinyl compounds (used as positive controls) is probably an inadequate indication that the activation system can metabolize halogenated alkanes such as CCl_4. A better indication that the activation system is sufficient may be to show that it metabolizes $^{14}CCl_4$ to intermediates that bind to macromolecules. It is also conceivable that potentially mutagenic reactive derivatives of CCl_4 (such as the free radical $\cdot CCl_3$ and phosgene) are generated in the presence of an S9 activation system but are too short-lived to interact with DNA in in vitro test systems.

The study by Callen et al. (1980) was designed to overcome this problem by using an in vivo activation system in yeast. Positive results for mutagenicity and recombinogenicity were reported. In contrast, negative results for DNA recombinogenicity were reported using in vivo UDS as the assay endpoint. These negative results should be confirmed, however, by the more sensitive in vitro hepatocyte UDS assay of Williams (1981). Binding studies by Rocchi et al. (1973) and by Diaz Gomez and Castro (1980a, 1980b, and 1981) indicate that metabolically activated CCl_4 may interact with DNA.

The negative genotoxicity test results that have been reported may be due to any of four (or more) factors: (1) CCl_4 is not mutagenic, (2) excessive volatilization and escape of CCl_4 if appropriate precautions are not taken, (3) inadequate activation of CCl_4 to a metabolite capable of causing mutations (e.g, $\cdot CCl_3$ or phosgene) by the S9 system, or (4) inability of reactive derivations of CCl_4 to reach DNA before being scavenged by lipid and protein (particularly under conditions of exogenous activation, such as in the Salmonella test). Additional testing should incorporate appropriate measures to ensure that (1) volatilization and escape of CCl_4 does not decrease exposure of the test organism or cell to ineffective levels, (2) metabolic activation is occurring, and (3) DNA is exposed to the activated chemical species.

In its totality, the evidence described in this report is insufficient to allow firm conclusions concerning the genotoxicity of CCl_4. The marginal results for binding of reactive intermediates to DNA (Rocchi et al. 1973; Diaz Gomez and Castro, 1980a and 1981), the study of Callen et al. in yeast (1980), and the questionable evidence for mutagenicity in the Salmonella assay (Cooper and Witmer, 1982) are insufficient evidence for genotoxicity. Since they do suggest the possibility of weak genotoxic effects, however, further studies should be done.

4.3. DICHLOROMETHANE

Dichloromethane (DCM) has been tested for mutagenic activity in bacteria, yeast, insects, nematodes, mammalian cells in vitro, and rodents. These studies are discussed below and are summarized in Tables 4-3 to 4-8.

4.3.1. Gene Mutation Studies

4.3.1.1. Bacteria--There are 14 reports in the literature concerning the mutagenic potential of DCM in bacteria; the Salmonella histidine reversion assay was used in all of these studies (Simmon et al., 1977; Simmon and Kauhanen, 1978; Kanada and Uyeta, 1978; Jongen et al., 1978, 1982; McGregor, 1979; Snow et al., 1979; Green, 1980, 1981; Rapson et al., 1980; Barber et al., 1980; Nestmann et al., 1980, 1981; Gocke et al., 1981). Kanada and Uyeta (1978) also tested DCM in the *Bacillus subtilis rec* assay. DCM was positive in all studies in Salmonella without or with metabolic activation in strains TA100, TA1535, or TA98 when assays were performed in sealed, gastight exposure chambers. Negative responses were reported by Rapson et al. (1980) and Nestmann et al. (1980) in standard assays, but these tests are inadequate because DCM was added directly to the agar medium and no precautions were taken to prevent excessive evaporation of the test material. The tests were carried out at 37°C, which is close to the boiling point of DCM (39°C), and it is very likely that excessive evaporation occurred. Data were presented in many of the reports, and a clear dose-related response is apparent for each. Tenfold or greater increases in numbers of revertants per plate were observed at the highest doses compared with negative controls. The doses employed and the responses observed are summarized in Table 4-3.

The purity of the test material was not given in any report. Because of this, most positive responses must be viewed with caution, because substances other than DCM may contribute to the observed mutagenicity. For instance,

TABLE 4-3. MUTAGENICITY TESTING OF DCM IN BACTERIA

Reference	Test system	Strain	Activation system	Concentration/Result		Comments
Simmon et al., 1977	Salmonella/ S9 vapor exposure	TA1535 TA1537 TA1538 TA98 TA100	None	(Extrapolated from Fig. 17) 0, 50, 100, 200, 400, and 800 ul/9 L desiccator	(Extrapolated from Fig. 17) Dose (ul) / TA100 Revertants/plate 0 / 170 50 / 210 100 / 300 200 / 400 400 / 650 800 / 1350	1. Toxicity not reported. 2. Number of revertants observed for TA100 not specified numerically. 3. Data not presented for strains other than TA100. 4. Purity and source of compound not provided. 5. Positive response.
Simmon and Kauhanen, 1978	Salmonella/ S9 vapor exposure	TA100	Aroclor-1254 induced rat liver microsome S9 mix	0 and 1 ml/9 L desiccator for 6.5 and 8 hours	No.h / S9 / TA100 revertants Treated / Control 6.5 / - / 688 / 133 + / 1344 / 130 8 / - / 830 / 174 + / 912 / 158	1. Toxicity not reported. 2. Purity and source of compound not provided. 3. Used as a positive control in the testing of 2-chloroethyl-chloroformate. 4. Positive response.

(continued on the following page)

TABLE 4-3. (continued)

Reference	Test system	Strain	Activation system	Concentration	Result	Comments
Kanada and Uyeta, 1978 Abstract	Salmonella/ S9 and *B. subtilis* *rec* assay testing	TA98 TA100	PCB-induced rat liver microsome S9 mix	Not reported	DCM reported negative in *B. subtilis* and positive for both strains in *S. typhimurium*	1. Results summarized in abstract form. 2. Positive results in Ames test supports reports by other authors using same system.
Jongen et al., 1978	Salmonella/ S9 vapor exposure	TA98 TA100	Phenobarbital-induced rat liver microsome S9 mix	(ppm x 10^3)	TA100 +S9 / TA100 -S9 / TA98 +S9 / TA98 -S9	1. Testing conducted in gastight perspex boxes. 2. Only highest dose exhibited less than 83% survival. 3. Purity of DCM not reported. 4. Positive response. 5. Results from 3 experiments, 5 plates per dose.
				0	152±19 / 129±12 / 21±4 / 19±5	
				5.7	329±37 / 248±32 / 54±5 / 44±8	
				11.4	515±76 / 407±47 / 74±4 / 56±10	
				14.1	757±82 / 582±56 / 93±9 / 66±12	
				22.8	865±82 / 653±89 / 123±10 / 96±11	
				57.0	1201±191 / 740±94 / 149±42 / 110±42	

(continued on the following page)

TABLE 4-3. (continued)

Reference	Test system	Strain	Activation system	Dose	Result				Comments
					TA100		TA98		
					+S9	-S9	+S9	-S9	
Snow et al., 1979	Salmonella/S9 vapor exposure	TA98 TA100	DCM-induced Syrian golden hamster liver S9 microsome mix	(ul/chamber)					1. Purity of DCM not reported. 2. No information about variability of results. 3. Positive response. 4. Mean calculated from 3 plates per dose.
				0	66	63	38	19	
				100	177	142	47	31	
				300	463	274	69	46	
				500	642	468	92	61	
				1000	972	632	39	72	

(continued on the following page)

TABLE 4-3. (continued)

Reference	Test system	Strain	Activation system	Concentration/Result				Comments
McGregor, 1979	Salmonella/S9 vapor exposure	TA1535	None	Atmospheric concentration % Theoretical	Actual	plate concentration (ug)	Revertants	1. Purity of DCM not reported. 2. Positive response.
				0	ND	ND	15	
				0.5	0.14	245	20	
				1.0	0.33	600,595,530	25	
				2.0	0.67	1400	50	
				4.0	1.60	2425	75	
				10	ND	ND	80	
Nestmann et al., 1980	Salmonella/S9 vapor exposure	TA1535 TA1537 TA1538 TA98 TA100	Aroclor-induced rat liver S9					1. Data not presented. 2. Negative response in standard test. 3. Positive response in gas-tight chamber. Doubling in revertant counts for TA1535; six-fold increase for TA100.

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TABLE 4-3. (continued)

Reference	Test system	Strain	Activation system	Dose	Result		Comments
				Concentration (% in air)	TA 100 +S9	 -S9	
Green, 1980	Salmonella/S9 vapor exposure	TA1535 TA100	Rat liver fractions	0	69±3	-	1. Preliminary results presented in abstract form. 2. Metabolic studies conducted in rat tissue and TA100. Similar metabolism in both systems. Radiolabel reported to bind to bacterial DNA but not to rat liver DNA. 3. Purity of DCM not reported. 4. Positive response. Green thinks that this is due to close proximity of cytoplasmic enzymes and intermediates to DNA in bacteria and that negative responses would be obtained in higher organisms. Positive responses in other tests argue against this. See discussion in section 4.3.1.1.
				1.4	283±10	267±20	
				2.8	506±27	462±28	
				5.5	825±34	872±27	
				8.3	1050±88	997±88	

(continued on the following page)

TABLE 4-3. (continued)

Reference	Test system	ppm Vapor	umol/plate	Revertants/Plate						Comments
				TA1535		TA98		TA100		
				-S9	+S9	-S9	+S9	-S9	+S9	
Barber et al., 1980	Salmonella/S9 vapor exposure	0	0	23	28	23	39	254	264	1. Tested redistilled sample of DCM > 99.9% pure. 2. Revertants/nmol at highest dose for TA1535, TA98, and TA100 were 0.0006, 0.006, and 0.03, respectively. 3. Data shown for testing in gastight chamber. 4. Negative response in standard test; positive response in gastight chamber. 5. DCM concentration measured by GC/MC in aqueous phase of petri dish.
		3,600	38	40	36	259	288	752	1152	
		7,200	76	59	51	441	297	1440	960	
		9,100	96	78	78	459	322	2640	1096	
		10,900	115	64	50	741	479	3060	3240	

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TABLE 4-3. (continued)

Reference	Test system	Strain	Activation system	Concentration/Result	Comments
Nestmann et al., 1981	Salmonella/S9 vapor exposure	TA1535 TA1537 TA1538 TA98 TA100	Aroclor 1254-induced rat liver S9		1. Levels of DCM in exposure chambers related directly to the mutational dose-effect curves of three paint removers. 2. Data shown for one paint remover only. Other two gave similar response. 3. Purity of DCM not reported. 4. Positive response for paint removers likely due to DCM.

Material			his+ Revertants/Plate[a]			Exposure level (mg/L)				
Type	Weight (mg)					DCM			Methanol	Ethanol[b]
	Added	Vaporized	TA1535	TA100	TA98	Time Averaged[c]	Max 6h Calculated[d]	Measured		
Paint remover	0	---	16	144	25	---	---	---	---	---
	203	144	22	310	31	12.7	15.5	13.0	<0.5	---
	370	241	14	433	42	21.9	25.5	23.0	<0.5	---
	790	469	23	563	76	40.1	49.9	45.0	0.7	---
	1435	903	31	785	60	80.2	95.4	86.0	1.9	---
		(mL)								
Mix (90:5:5 v/v/v DCM/methanol/ethanol)	0	---	13	154	32	---	---	---	---	---
	0.1	0.1	15	268	43	12.2	12.8	11.5	<0.5	<0.9
	0.2	0.2	24	401	73	26.9	25.3	27.5	<0.5	0.7
	0.4	0.4	25	789	138	50.6	50.8	50.0	0.9	1.1
	0.8	0.8	34	1084	164	94.1	101.0	94.5	2.6	2.7

(continued on the following page)

[a] Average values from triplicate plates.
[b] Maximum measured.
[c] Determined from an area under curve for concentration against time.
[d] Calculated from amount vaporized assuming only DCM vaporized in 9 L chamber.

TABLE 4-3. (continued)

Reference	Test system	Strain	Activation system	Dose	Result	Comments
Green, 1981	Salmonella/S9 vapor exposure	TA100	Aroclor-1254 induced rat liver S9, microsomes, and cytosol	% Vapor 0 2.8 5.0 8.4	Revertants +S9 -S9 100 100 458 386 700 720 950 900	1. Bacterial and mammalian metabolism similar. 2. Cytosol and glutathione catalyze DCM to formaldehyde and CO_2. S-chloromethylglutathione is a putative intermediate. 3. DCM converted to carbon monoxide in the presence of microsomes. Formyl chloride is a putative intermediate. 4. Purity of DCM not given. 5. Positive response. See table entry for Green, 1980.
Gocke et al., 1981	Salmonella/S9 vapor exposure	TA1535 TA1537 TA1538 TA98 TA100	Aroclor-1254 rat liver S9	ul/desiccator 0 125 250 500 750	Revertants +S9 -S9 30±0 40±0 54±7 85±7 68±32 110±14 105±7 195±21 203±32 295±7	1. Spontaneous revertants for TA100 too low. 2. No information presented for toxicity. 3. Purity of DCM not given. 4. Positive response.
Jongen et al., 1982	Salmonella/S9 vapor exposure	TA100	Aroclor-1254 rat liver S9, microsomes, and cytosol	Activation --- S9 Cytosol Microsomes	% DCM 0 0.35 0.7 1.4 150 210 350 550 150 240 410 730 150 220 420 810 150 215 380 610	1. Purity of DCM not given. 2. Positive response.

formaldehyde, a metabolite of DCM, could form nonenzymatically by hydrolysis of DCM in the aqueous solutions used for biological testing (March, 1977). Because of the consistency of positive responses with several different samples, however, it is highly likely that the DCM itself is mutagenic. For instance, in their tests of DCM, Barber et al. (1980) used a redistilled sample of DCM estimated to be >99.9% pure, containing only traces of 1,1- and 1,2-dichloroethane, chloroform, chloromethane, and an unidentified C_5H_{10} aliphatic material (Dr. E. Barber, Eastman Kodak, personal communication). Nestmann et al. (1981) reported that their sample was "gas chromatographically pure," and Gocke et al. (1981) checked their sample for the "correct melting point (sic) and elementary analysis." The consistency of the positive responses indicate that the mutagenicity in Salmonella can be attributed to DCM. Barber et al. (1980) conducted their tests in a chemically inert, closed incubation system and analyzed the concentrations of DCM in the vapor-phase head space and in the aqueous phase of a test plate by gas-liquid chromatography (Barber et al., 1981). The mutagenic responses at the highest dose (i.e., 115 umol/plate) for TA1535, TA98, and TA100 were 0.0006, 0.006, and 0.03 revertants per umol, respectively, indicating that DCM is a weak mutagen for Salmonella under the conditions of the test.

Although the results clearly show that DCM is mutagenic in Salmonella, questions have been raised about their applicability to predicting mutagenicity in other species, especially mammals. DCM is metabolized, apparently via mutagenic intermediates, to CO and CO_2 in both rodents and humans. CO is produced by oxidative dechlorination of DCM by the microsomal P-450 mixed-function oxidase system. Formyl chloride is believed to be an intermediate in this pathway. A second cytosolic glutathione transferase system dehalogenates DCM to produce formaldehyde, which is further oxidized to CO_2. This pathway is thought to proceed via an S-chloromethyl glutathione intermediate (Ahmed and Anders, 1978; Kubic and Anders, 1975). Formyl chloride and S-chloromethyl glutathione are highly reactive alkylating agents. Salmonella also metabolizes DCM to CO_2 and CO, apparently by reaction pathways similar to those occurring in mammals (Green, 1980, 1981, and 1983).

Because of the reactivity of formaldehyde, formyl chloride, and S-chloromethyl glutathione and the proximity of bacterial DNA to bacterial cytoplasmic enzymes, it has been hypothesized that these chemical substances are more effective as mutagens when they are formed by bacterial metabolism than when they are formed outside the bacterial cell by rat liver fractions (Green, 1980, 1981, and 1983). The basis for this hypothesis is that rat liver fractions used for metabolic activation have little effect on the mutagenicity of DCM in the Ames test. The implication is made that as organismic complexity is increased, there is less likelihood of DCM causing mutations. It is argued that compartmentalization of DNA into the nucleus protects the genetic material from exposure to the mutagenic metabolites of DCM (i.e., they would react with other cellular constituents first) so there is little or no mutagenic risk. While the intracellular compartmentalization of eucaryotes may reduce the frequency with which short-lived, highly reactive metabolites interact with DNA, the positive results discussed in the following paragraphs show that genetic damage occurs in eucaryotes exposed to DCM as well.

4.3.1.2. Yeast--Callen et al. (1980) studied the ability of DCM obtained from Fisher Scientific Company (purity not reported) and six other halogenated hydrocarbons to cause gene conversion, mitotic recombination, and reverse mutations in *S. cerevisiae* (Table 4-4). Strain D7 log phase cells were incubated for 1 hour in culture medium containing 0, 104, 157, and 209 mM DCM. The percent survival for these doses was 100, 77, 42, and <0.1, respectively.

TABLE 4-4. GENE MUTATIONS AND MITOTIC RECOMBINATION IN YEAST

Reference	Test system	Strain	Dose (mM)	Survival %	Response/10^6 survivors				Comments
						ade-2			
					trp-5 Conversion	Mitotic crossing over	Total genetic alterations	ilv-1 Revertants	
Callen et al., 1980	*Saccharomyces cerevisiae*	D7	0	100	18	310	3,300	2.7	1. Positive response. 2. Active metabolites produced by this system are made intracellularly rather than by an exogenous activation system.
			104	77	28	190	3,900	4.4	
			157	42	107	4490	14,000	5.8	
			209	--	--	---	---	---	
Simmon et al., 1977	*Saccharomyces cerevisiae* suspension test	D3							1. Data not provided, but reported negative for mitotic recombination. 2. Strain differences and differences in treatment conditions (i.e., time and temperature) may be the cause of differences between this study and that of Callen et al. (1980). 3. Cytochrome P-450 concentration not known. Callen et al. (1980) report different yeast strains have different levels.

Due to the toxicity of the compound, the genetic endpoints were not measured at the highest dose. The responses for the other doses (0, 104, and 157 mM) expressed per 10^6 survivors were: gene conversion at the trp-5 locus (18, 28, and 107); mitotic recombination for ade-2 (310, 190, and 4,490); total genetic alterations for ade-2 (3,300, 3,900, and 14,000); and reverse mutations for ilv-1 (2.7, 4.4, and 5.8). A greater than twofold dose-related increase over negative controls was observed for each endpoint measured. The magnitude of the recombinogenic response at the ade locus may have been overestimated in this study because the treatment regime used for estimating the recombinants overlaps that used for estimating the number of $trp\text{-}5^+$ convertants. No exogenous metabolic activation was used in these experiments, which indicates that yeast cells metabolize DCM intracellularly to a mutagenic intermediate(s) that reaches nuclear DNA. Simmon et al. (1977) reported that DCM (source and purity not given, but stated to be the highest available purity) did not induce mitotic recombination in strain D3 of *S. cerevisiae* when cells (1×10^8) in suspension culture were exposed for 4 hours at 30°C (Table 4-4). The doses and the experimental values obtained for mitotic recombination were not reported. The discrepancies between the work by Callen et al. (1980) and Simmon et al. (1977) may be due to a number of factors including the different strains used (D3 vs. D7), exposure time differences (4 hours vs. 1 hour), or differences in the incubation temperature (30°C vs. 37°C). Callen et al. (1980) reported that increasing the treatment time from 1 hour to 4 hours significantly reduced the genetic activity detected in strain D7. Other variables, such as a lower level of cytochrome P-450 enzymes in strain D3, could also account for the discrepancy in the results.

On the basis of the results in strain D7, DCM is considered to be a mutagen and recombinogen in yeast.

4.3.1.3. Drosophila--Two reports are available concerning the ability of DCM to induce sex-linked recessive lethal mutations in *D. melanogaster* (Table 4-5). Abrahamson and Valencia (1980) reported negative results, whereas a positive response was reported by Gocke et al. (1981). Abrahamson and Valencia (1980) conducted their sex-linked recessive lethal tests using two routes of administration, adult feeding and injection. Due to the low solubility of DCM in aqueous solutions, high concentrations of the test substance were not used in these experiments, which may account for the negative response observed. In the feeding study, Canton-S males were placed in culture vials containing glass microfiber paper soaked with a saturated solution of 1.9% DCM (224 mM) in a sugar solution for 3 days. The feeding solution was added twice daily to compensate for evaporation of the compound. At this dose, there was no evidence of toxicity. After mating, chromosomes from the 14,682 offspring of treated parents and chromosomes from the 12,450 offspring of concurrent control parents were assessed for recessive lethal mutations. No evidence of mutagenicity was observed. DCM gave a level of 0.204% lethal mutations compared with 0.215% in controls. Because of the volatility and insolubility of DCM, the actual dosages may have been less than expected.

In the injection study, 0.3 ul of an isotonic solution containing 0.2% DCM was administered to male flies. This exposure level resulted in 30% post-injection mortality. The post-injection mortality observed for the controls was not reported. This is a critical omission because the mortality in injection studies is due not only to the test chemical, but also to the damage caused by injection, and concurrent negative controls are necessary to reach conclusions about the effects of the treatment. After mating, 8,262 chromosomes from the offspring of treated parents and 8,723 chromosomes from the offspring of control

TABLE 4-5. GENE MUTATIONS IN MULTICELLULAR EUCARYOTES IN VIVO

Reference	Test system	Strain	Chemical	Route	Numbers of Chromosomes tested	Numbers of Lethals	Corrected (%)lethals	Comments
Abrahamson and Valencia, 1980	Drosophila sex-linked recessive lethal test	FM6 females, Canton S males	EMS	AF[a]	773	44	5.69	1. No exposure chambers designed to prevent evaporation of the compound in feeding experiments. 2. No concurrent negative controls reported for the injection experiment. 3. Negative response at dose tested (224 mM).
			Tris-BP	AF	2,442	35	1.43	
			Neg. controls	AF or AI[b]	94,491	230	0.233	
			DCM	AF	14,682	34	0.204	
				AI	8,262	18	0.157	
Gocke et al., 1981	Drosophila sex-linked recessive lethal test	Basc females, Berlin-K males	DCM (mM)	Lethals/Brood 1	2	3	Total treated	1. Positive response for Brood 1 indicating DCM is mutagenic in sperm of Drosophila. 2. Higher dose used than in test by Abrahamson and Valencia (1980).
			0	19/7130 (0.27)	8/5525 (0.14)	13/3416 (0.38)	40/16071 (0.25)	
			125	16/3632 (0.44)	2/2579 (0.08)	6/1310 (0.46)	24/7521 (0.32)	
			620	8/1213 (0.66)	3/735 (0.41)	5/1005 (0.50)	16/2953 (0.54)	
							$X^2 = 3.17$ $P < 0.05$	

(continued on the following page)

[a] Adult feeding
[b] Adult injection

TABLE 4-5. (continued)

Reference	Test system	Concentration (mol/L)	Mutation frequency (Lethal mutations/10^5 loci)	Toxicity (Survival rel. to controls)				Comments
				L2 Juveniles	L2-L3 Molt	L3-L4 Molt	L4 Adult Molt	
Samoiloff	*Panagrelus*	DCM						1. Equivocal response 2. No dose-related effects. 3. Some positive controls gave negative (e.g., EMS) or only marginally positive (e.g., 3-methylcholanthrene) response. 4. Test system not validated.
		0	2.2					
et al.,	*redivivus*	10^{-8}	6.0	1.02	0.99	0.97	0.46	
1980	sex-linked	10^{-6}	10.1	1.02	1.00	0.86	0.15	
	recessive	10^{-4}	9.8	1.00	1.00	0.88	0.17	

parents were assessed for recessive lethal mutations. No evidence of mutagenicity was observed by this route of administration. Flies injected with 0.2% DCM had 0.157% lethals compared to 0.206% for controls.

Gocke et al. (1981) also tested DCM (Merck, Darmstadt, FRG, purity not given) for its ability to induce sex-linked recessive lethal mutations in Drosophila. Two solutions, 125 mM and 625 mM in 2% DMSO and 5% saccharose, were fed to wild-type Berlin-K male flies for an unreported period of time. The highest dose (620 mM) is reportedly close to the LD_{50}. The males were then mated to Basc females and, three broods were scored (i.e., offspring from virgin females mated to treated males on days 1 through 3, 4 through 6, and 7 through 10 after exposure). There was a dose-related increase in lethals. Results shown in Table 4-5 indicate that DCM is not only positive but appears to induce a dose-related increase (see data from brood I), providing strong evidence that the compound is mutagenic in germ cells of an in vivo multicellular eucaryote test system. The discrepancy between the results of Abrahamson and Valencia (1980) and Gocke et al. (1981) are likely due to the fact that larger doses of DCM were employed by Gocke et al. (1981).

4.3.1.4. Nematodes--In another sex-linked recessive lethal test, Samoiloff et al. (1980) tested DCM for its ability to induce mutations in the nematode Panagrellus redivivus (Table 4-5). Individual females homozygous for the X-linked mutation b7 (coiled phenotype in liquid medium) were grown for 120 h in the presence of several concentrations of DCM ranging from 10^{-8} to 10^{-3}M. They were then washed and mated to S-15 males that carry an X-chromosome inversion extending at least 15 recombination units to either side of b7. One hundred female progeny were collected and mated to wild-type (C-15) males, and their progeny were scored for the presence of the b7 phenotype. The absence of b7 male progeny indicates lethality of the X-chromosome marked with b7 derived from a female grown on DCM. Three replicate experiments were performed. A non-dose-related increase in the level of lethals was observed in the progeny of DCM-treated worms compared with the negative controls. For worms treated with 10^{-8}, 10^{-6}, and 10^{-4}M DCM, the corresponding lethal mutations/10^5 loci were 6.0, 10.1, and 9.8, respectively, compared with an estimated spontaneous mutation frequency of 2.2×10^{-6} mutations per locus. Some of the positive controls tested concurrently, such as proflavine, yielded a positive response (12.5, 10.0, and 28.6 lethals/10^5 loci at 10^{-8}, 10^{-6}, and 10^{-4}M, respectively); but others, such as aflatoxin B and ethyl methanesulfonate (EMS), did not cause an increase in lethal mutations. The investigators suggest that DCM is mutagenic in nematodes, but firm conclusions cannot be made because the assay is not validated and because negative responses were obtained with some of the positive controls, especially EMS.

4.3.1.5. Mammalian cells in culture--Jongen et al. (1981) tested DCM for its mutagenic potential in several tests in cultured mammalian cells. Tests for the induction of forward mutations at the HGPRT locus are described here (Table 4-6), whereas tests for the ability of DCM to cause sister chromatid exchange (SCE), unscheduled DNA synthesis (UDS), and inhibition of DNA synthesis (IDS) are discussed in section 4.3.3. on other studies indicative of DNA damage.

To determine whether DCM induces forward mutations in cultured Chinese hamster cells, Jongen et al. (1981) incubated log phase CHO up to 5% DCM and V79 cells up to 4% DCM at 37°C for 1 hour in a closed glass container without S9 mix. Analytical grade DCM was obtained from Merck. The cells were exposed to gaseous DCM and then DCM in solution for 15-minutes intervals each by alternately tilting the plates and then placing them horizontally. After growth to allow

TABLE 4-6. GENE MUTATIONS IN MAMMALIAN CELLS IN CULTURE

Reference	Test system	Concentration (%)	V79		CHO		Comments
			Mutants/10^5 survivors	Survival (%)	Mutants/10^5 survivors	Survival (%)	
Jongen et al., 1981	6-Thioguanine resistance in V79 and CHO cells	DCM 0	2	100	1.9	100	1. Equivocal negative reponse. 2. Highest dose resulted in 20-25% decrease in survival. Higher doses should be tested.
		1	1.8	98	1.8	90	
		2	2	95	1.2	85	
		3	1.7	85	0.9	80	
		4	1.6	80	2.1	73	
		5			2.5	76	

for an 8-day (CHO cells) or 6-day (V79 cells) expression period, mutant cells were selected in thioguanine-containing medium. DCM failed to increase the mutation frequency of either cell line at any dose. The positive control, EMS, yielded a dose-dependent increase in mutation induction in V79 cells, but was not tested in CHO cells. DCM was not very cytotoxic to either cell line; at the highest dose, survival decreased by only 20-25%. It would be appropriate to repeat the experiment using higher doses of DCM.

Based on the positive responses in bacteria, yeast, and Drosophila, and the suggested positive in the nematode Panagrellus, DCM is capable of inducing gene mutations. Metabolic activation to highly reactive mutagenic metabolites apparently accounts for this response; and although these are thought to be short-lived unstable intermediates, they appear capable of interacting with the genetic material of both procaryotes and eucaryotes.

4.3.2. Cytogenetic Studies

Three studies on the ability of DCM to cause chromosomal aberrations were evaluated. Burek et al. (1984) subjected four groups of 10 Sprague-Dawley albino rats (Spartan substrain, SPF-derived, 5 males and 5 females) to 0, 500, 1,500, or 3,500 ppm DCM by inhalation 6 hours per day, 5 days/week, for 6 months. The animals were then sacrificed, bone marrow cells collected, chromosome preparations made, and slides coded and analyzed. Two hundred metaphases per animal were scored and aberrations were tabulated (Table 4-7). No increase in the total frequency of abnormal cells or in the frequency of any specific type of aberration was reported in the treated compared with the control animals. There were 1.1 ± 1.3, 0.6 ± 0.7, 0.8 ± 1.2, and 1.1 ± 0.9% cells with chromosome aberrations in animals treated with 0, 500, 1,500, and 3,500 ppm DCM, respectively.

TABLE 4-7. TESTS FOR CHROMOSOMAL ABERRATIONS

Reference	Strain/tissue	Route of exposure	Dose (ppm)	Breaks Chromatid	Breaks Chromosome	Dicentrics	Rings	Exchanges	Comments
Burek et al., 1984	Male and female Sprague-Dawley rat/bone marrow	Inhalation	0 500 1500 3500	0.9 ± 0.99 0.5 ± 0.71 0.5 ± 0.97 0.7 ± 0.48	0.2 ± 0.42 0.2 ± 0.42 0.1 ± 0.32 0.2 ± 0.42	0 0 0 0	0 0 0 0.2 ± 0.42	0 0 0.1 ± 0.32 0	1. 5 animals/sex/dose. 2. 200 cells/animal. 3. Dose to bone marrow cells may have been low. 4. Negative response.

Reference	test system	Dose	RCG[a]	Breaks Chromatid	Breaks Isochromatid	Exchange	Number of aberrations/cell	% Aberrant cells	Comments
Thilager and Kumaroo, 1983	Cultured CHO cells	DCM (ul/ml)							1. Positive response. 2. Four experiments yielded similar response.
		0	100	2	0	0	0.02	2	
		2	98.4	4	0	2	0.06	6	
		5	75.3	8	14	8	0.34	26	
		10	66.7	12	34	10	0.56	38	

[a]RCG = Relative cell growth.

Reference	Strain/tissue	Route of exposure	Dose (ppm) No. injection x mg/kg	Micronuleated polychromatic erythrocytes (%)	Comments
Gocke et al., 1981	Male and female NMRI mice/bone marrow	i.p. Injection	0 2 x 425 2 x 850 2 x 1700	0.19 0.19 0.35 0.28	1. Suggestive of positive response; authors interpret as negative response. 2. Dose to bone marrow cells may have been low.

Thilagar and Kumaroo (1983) treated CHO cells grown in either plastic or glass culture flasks with 0, 2, 5, 10, and in one experiment 15 ul/ml (i.e., 0, 31, 78, 156, and 234 mM) DCM for 2 hours with or 12 hours without S9 mix derived from Aroclor-induced rat livers. DCM was obtained from Fisher Scientific (certified A.C.S., lot no. 713580). After the exposure period, the cells were washed, placed in fresh media and allowed to grow before being arrested at metaphase with colcemid and harvested for chromosome preparation. Slides were coded and read "blind"; 100 cells were scored for each dose level (50 cells per duplicate flask). DCM induced a dose-related increase in chromosome aberrations (Table 4-7) ranging from 0.02 aberrations per cell in the negative controls to 1.44 aberrations/cell at 15 ul/ml (234 mM). The response was not dependent on the presence of the exogenous metabolic activation system.

Gocke et al. (1981) assessed the ability of DCM (Merck, Darmstadt; purity not given) to cause micronuclei in polychromatic erythrocytes (PCE). Two male and two female NMRI mice were used for each of three dose levels (425, 850, and 1,700 mg/kg per intraperitoneal injection). The highest dose approximated the LD_{50} for mice. Intraperitoneal injections of each dose were given at 0 and 24 hours, the animals were sacrificed at 30 hours, bone marrow smears were made, and 1,000 PCEs per animal were scored for the presence of micronuclei. An increase in PCEs with micronuclei was observed at the two highest doses, but the response was not dose-related and was not double the control value. There were 0.19% micronuclei in the untreated controls compared with 0.35% micronuclei in the animals receiving two injections of 850 mg/kg, and 0.28% micronuclei at the highest dose. The results are therefore suggestive of a positive response, but are not conclusive.

Based on the positive response reported by Thilagar and Kumaroo (1983), DCM is tentatively judged to be capable of causing chromosomal aberrations. The negative responses reported by Burek et al. (1984) and Gocke et al. (1981) are not inconsistent with these results. Thilagar and Kumaroo (1983) exposed mammalian cells in culture to DCM. The studies by Burek et al. (1984) and Gocke et al. (1981) where exposure occurred in vivo are not comparable to the in vitro studies.

4.3.3. Other Studies Indicative of DNA Damage

4.3.3.1. Sister Chromatid Exchange (SCE)--Two papers and one abstract have been published on the ability of DCM to induce SCEs (Table 4-8). Jongen et al. (1981) tested the ability of 0.5, 1.0, 2.0, 3.0, and 4% DCM (i.e., 58, 118, 235, 353, and 471 mM) to induce SCEs in V79 cells. Log phase cells were incubated at 37°C for 1 hour in a closed glass container. The cells were exposed to DCM in the gaseous phase and in the medium by tilting the plates for 15 min and then placing them horizontally. The experiment was conducted 7 times and each yielded a dose-related increase in SCEs per cell, which approached but did not exceed a twofold increase above the control level. An analysis of variance of effects of different doses within experiments showed the increases in frequency of SCEs to be statistically significant ($P < 0.001$). Increasing the exposure time to 2 hours or 4 hours or using S9 from rat liver did not alter the shape of the dose-response curve, which reached a plateau at 1% DCM. Jongen et al. suggest that this phenomenon is due to a saturation of the metabolic activation system of V79 cells.

Thilagar and Kumaroo (1983) exposed CHO cells to 0, 2, 5, 10, and in one experiment 15 ul DCM/ml of medium (0, 31, 78, 156, and 234 mM DCM) for 2 hours with and 24 hours without metabolic activation. The cells were grown for 24 hours in BrdUrd followed by a mitotic shake-off, fixing, and staining by a

TABLE 4-8. TESTS FOR SISTER-CHROMATID EXCHANGE

Reference	Test system	Dose	Results		Comments
		% DCM	SCE/cell experiment # 1	7	
Jongen et al., 1981	V79 cells in culture	0	0.26 ± 0.02	0.03 ± 0.03	1. Exposure time 1 h. 2. Positive response. Significant increases in SCEs ($P < 0.001$). 3. Same type of dose response observed in experiments 2-6 (data not shown). 4. DMSO did not increase the frequency of SCE.
		0.5	0.40 ± 0.02	---	
		1.0	0.46 ± 0.02	0.47 ± 0.02	
		2.0	0.45 ± 0.03	0.51 ± 0.03	
		3.0	---	0.58 ± 0.03	
		4.0	0.51 ± 0.03	0.61 ± 0.03	

(continued on the following page)

TABLE 4-8. (continued)

Reference	Test system	Dose	SCE/Cell ($X \pm SD$)	Range of SCEs	M_1	$M_1 + M_2$		Comments
Thilagar and Kumaroo, 1983	CHO cells	DCM (ul/ml) 0	10.28 ± 3.17	5-17	0	3	97	1. Marginal, but not significant increases in SCEs. 2. Three other experiments yielded similar responses. 3. Results not inconsistent with test by Jongen et al. (1981) where highest dose was three times greater (i.e., 471 mM vs. 156 mM).
		2	11.36 ± 3.09	3-19	0	16	84	
		5	12.56 ± 2.95	7-18	6	54	40	
		10	12.36 ± 3.35	7-21	4	56	40	

fluorescence-plus-Giemsa technique. The coded slides were scored "blind." Slight dose-related elevations in SCE values were noted (Table 4-8), but they never exceeded a 50% increase at the highest dose. Thilagar and Kumaroo judged their test to be negative.

McCarroll et al. (1983) reported in an abstract that consistent and dose-related increases were observed in SCEs in CHO cells following 24-hour exposures to 1, 3.6, 5.4, and 7% atmospheres of DCM. A 7% atmosphere was required to elicit a statistically significant increase. Based on the reports of Jongen et al. (1981), Thilagar and Kumaroo (1983), and McCarroll et al. (1983), DCM is capable of causing DNA damage that results in SCE.

4.3.3.2. DNA repair assays--Jongen et al. (1981) measured UDS and inhibition of DNA synthesis (IDS) in V79 cells and primary human fibroblasts (AH cells). These experiments were conducted by exposing 10^5 cells attached to glass coverslips (UDS assay) or to glass petri plates (IDS assay) to 0.5, 1.0, 2.0, 3.0, and 5.0% DCM (58, 118, 235, 353, and 471 mM, respectively) without metabolic activation. UDS experiments were done in duplicate, and at least 25 nuclei of non-S phase cells were scored for the number of silver grains per nucleus at each dose level. DCM had no detectable effect on UDS in either cell line. In the IDS assays, the relative rate of DNA synthesis was determined radioisotopically immediately after DCM exposure and 0.5, 1.5, and 3.5 hours later. The average of duplicate samples revealed that DCM inhibited DNA synthesis in V79 and AH cells at all dose levels compared with controls but that synthesis recovered with time after exposure in all cases. This is unlike the persistent inhibition of DNA synthesis by the positive control 4-nitroquinoline-1-oxide. The investigators conclude that DCM was not inducing genetic damage in cells but was inhibiting DNA synthesis by an effect on cell metabolism.

Perocco and Prodi (1981) also performed a UDS assay using DCM. Blood samples were collected from healthy individuals, the lymphocytes were separated, and cultured 5 times 10^5 cells in 0.2-ml medium for 4 hours at 37°C in the presence or absence of DCM (Carlo Erba, Milan, Italy or Merck-Schuchardt, Darmstadt, FRG, 97 to 99% pure). The tests were conducted with and without PCB-induced rat liver S9 mix. No difference was noted between the treated and control groups of cells with respect to scheduled DNA synthesis measured as dpm of [^{3}H] deoxythymidylic acid (TdR) after 4 hours of culture (2,661 ± 57 dpm in untreated cells compared with 2,356 ± 111 dpm in cells treated with 5 ul/ml [78 mM] DCM). Subsequently, 2.5, 5, and 10 ul/ml (39, 78, and 156 mM) DCM was added to cells cultured in 10 mM hydroxyurea to suppress scheduled DNA synthesis. The amount of unscheduled DNA synthesis was estimated by measuring dpm from incorporated [^{3}H]TdR 4 hours later. At 10 ul/ml DCM, 532 ± 31 and 537 ± 39 dpm were counted without and with exogenous metabolic activation, respectively. Both values were lower than the corresponding negative control values of 715 ± 24 and 612 ± 26 dpm, respectively. No positive controls were run to ensure that the system was working properly, although tests of chloromethyl methyl ether (CMME) with activation resulted in a doubling of dpms over the corresponding negative control values (1,320 ± 57 at 5 ul/ml CMME vs. 612 ± 26 untreated). The investigators calculated an effective DNA repair value (r) for each chemical based on the control and experimental values with and without metabolic activation. Perocco and Prodi (1981) evaluated DCM as negative in the test, but they did not state their criteria for classifying a chemical as positive. None of the experimental values from cells treated with DCM had higher dpm values than the controls.

Based on these experiments there is no evidence that DCM specifically inhibits DNA synthesis or causes UDS. Certain kinds of DNA alterations that lead to mutation either do not stimulate repair processes at all or do so to such a small extent that detection is not practical (Larsen et al., 1982).

4.3.4. Summary and Conclusions

Dichloromethane has been tested for its ability to cause gene mutations (in Salmonella, yeast, Drosophila, Panagrellus, and cultured mammalian cells), chromosomal aberrations (in rats, mice, and cultured mammalian cells), and other indicators of DNA damage in cultured cells (sister chromatid exchange, unscheduled DNA synthesis, and inhibition of DNA synthesis).

Commercially available samples of DCM have been shown to be mutagenic in a wide range of organisms, including bacteria (Salmonella), fungi (Saccharomyces), and insects (Drosophila). The responses were weak under the treatment conditions used and were obtained without the addition of exogenous metabolic activation systems (e.g., S9 mix). The data suggest that DCM is metabolized in vivo in various organisms to form mutagenic metabolite(s). Some negative results have been reported in tests for mitotic recombination in fungi (Saccharomyces) and gene mutations in cultured mammalian cells, but these may be false negatives because of the treatment conditions used. DCM has also been reported to induce chromosomal aberrations in cultured mammalian cells but not in bone marrow cells of animals exposed in vivo, perhaps because a sufficient dose of DCM did not reach the bone marrow. No tests have been conducted to assess the ability of DCM to cause chromosome nondisjunction. DCM causes a weak increase in SCEs, but has not been shown to cause UDS or to inhibit DNA synthesis.

Mutagenicity tests of DCM have given positive responses in four different organisms based on the weight of available evidence. DCM is judged to be a mutagen with the potential of inducing gene mutations in exposed human cells. A positive response in cultured mammalian cells indicates that it also causes chromosomal aberrations, but additional testing in another in vivo or in vitro chromosomal aberration assay is needed to confirm the available data. If such tests are conducted, care should be taken to ensure that the test cells are exposed to sufficiently high doses of DCM, otherwise false negative responses may be obtained. The magnitude of the mutagenic responses obtained with DCM is much less than those obtained for similar endpoints after treatment with ethylene dibromide (EDB).

4.4. ETHYLENE DICHLORIDE (1,2-DICHLOROETHANE)

Ethylene dichloride (EDC) has been tested for mutagenic activity in bacteria, plants, Drosophila, mammalian cells in vitro, and intact rodents. These studies are discussed below and are summarized in Tables 4-9 to 4-16. The reader may also refer to published reviews of the mutagenic potential of EDC (e.g., Fishbein, 1976, 1979; Fabricant and Chalmers, 1980; Rannug, 1980; Simmon, 1980).

4.4.1. Gene Mutation Studies

4.4.1.1. Bacteria and Fungi--Many investigators have studied the ability of EDC to produce gene mutations in bacteria (Table 4-9). Most of them reported marginal positive responses without metabolic activation and stronger positive responses with exogenous hepatic metabolic activation, indicating that EDC is weakly mutagenic but that metabolites, such as S-(2-chloroethyl)-L-cysteine, are more potent mutagens.

Ethylene dichloride has been reported positive in four Salmonella/microsome plate incorporation assays (McCann et al., 1975; Rannug, 1976; Rannug and Ramel, 1977; Rannug et al., 1978), in assays testing the mutagenicity of bile obtained from EDC-perfused rat livers or livers from EDC-treated mice (Rannug and Beije, 1979), and in two Salmonella spot tests. In one of the Salmonella spot tests (Brem et al., 1974), duplicate experiments carried out at different times revealed a reproducible twofold increase in revertant counts for strains TA1530 and TA1535 (mean values of 50 and 54 revertants on treated plates vs. 23 and 26 in control plates for TA1530 and TA1535, respectively). No difference in revertant counts was noted in strain TA1538. This response is consistent with that expected for an alkylating agent. Brem et al. stated that plate incorporation tests could not be performed because of the volatility of the

TABLE 4-9. SUMMARY OF MUTAGENICITY TESTING OF EDC: GENE MUTATIONS IN BACTERIA AND FUNGI

Reference	Test system	Strain	Activation system	Chemical information	Results	Comments
Brem et al., 1974	*Salmonella typhimurium* (spot test)	TA1530 TA1535 TA1538	None	10 umol on filter disk Source: Not given Purity: Not given	Weak positive	1. Could not perform plate incorporation tests because of volatility.

	Salmonella revertants		
	TA1530	TA1535	TA1538
EDC	50	54	19
Water	23	26	19
Chloramphenicol	20	31	14

(continued on the following page)

TABLE 4-9. (continued)

Reference	Test system	Strains	Dose ul/plate				Comments
			-S9		+S9		
			0	100	0	100	
Principe et al., 1981	Salmonella/ microsome assay (spot test)	TA1535	39	46	33	103^a	1. Positive controls indicated system working properly 2. Positive results in Salmonella in spot test with TA1535. 3. No precautions taken to prevent excessive evaporation of EDC and ensure adequate exposure. 4. Toxicity results indicate exposure was minimal.
		TA1537	17	8	10	8	
		TA1538	19	12	21	27	
		TA98	82	83	77	84	
		TA100	188	188	171	169	
	Streptomyces coelicolor forward mutation assay to Str^r		Dose ul/plate	Survival %	Str^r/plate		
			0	100	2.5 ± 0.6		
			2	100	0.2 ± 0.2		
			10	100	0.7 ± 0.4		
			20	100	0.7 ± 0.4		
			100	100	1.0 ± 0.8		
					8-AG/plate		
	Aspergiellus nidulans forward mutation to $8\text{-}AG^r$		0	100	2.5 ± 0.9		
			250	100	2.0 ± 1.1		
			500	42	1.0 ± 0.7		

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[a] $p < 0.01$.

TABLE 4-9. (continued)

Reference	Test system	Strain	Activation system	Chemical information	Results	Comments
McCann et al., 1975	Salmonella/ microsome assay (plate test)	TA100	PCB-induced rat liver/S9 mix	Concentration tested: 1.3 X 10^4 ug/plate (13 umol) Source: Aldrich Chemical Co. Purity: Not given, but stated to be highest purity	Negative or at best marginal positive response. Induced 25 colonies/ plate above background. (0.19 revertants/umol in TA100)	1. Nonmutagenic or extremely weak mutagen in this study. Reproducible dose-response curves not obtained. 2. Metabolic activation did not increase positive response. 3. Chloroethanol and chloroacetaldehyde (two putative intermediates in the metabolism of EDC in mammals) were positive (0.06 and 746 revertants/umol, respectively).

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TABLE 4-9. (continued)

Reference	Test system	Strain	Activation system	Chemical information	Results	Comments
Rannug, 1976	Salmonella/ microsome assay (plate test)	TA1535	Liver fractions from Sprague-Dawley or R strain Wistar rats induced with phenobarbital with and without NADPH-generating system and with and without glutathione S-transferases A, B, and C	Concentration tested: Up to 60 umol/plate Source: BDH Chemicals, Ltd. Purity: Not given but reported to be checked by glass capillary column chromatography using a flame ionization detector	Marginally positive without activation (two-fold increases); positive response with activation (ten-fold increases). Spontaneous background 8-14 revertants/ plate.	1. EDC activated by the liver cytosol fraction; mixed-function oxygenases not involved. 2. NADPH-independent GSH S-transferase dependent activation. 3. Strain differences noted in ability to metabolize EDC. 4. Thought that mutagenicity of EDC after activation caused by formation of highly reactive half sulfur mustard, S-(2-chloroethyl)-L-cysteine.

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TABLE 4-9. (continued)

Reference	Test system	Strain	Activation system	Chemical information	Results	Comments
Rannug and Ramel, 1977	Salmonella/ microsome assay (plate test)	TA1535	S9 mix from livers of uninduced male R strain Wistar rats plus NADPH-generating system	Concentration tested: Up to 45 umol/plate Source: BDH Chemicals, Ltd. Purity: Not given	Positive response (two-fold increase) without activation; nearly ten-fold increase with activation. Negative controls yielded roughly 15 revertants/plate.	1. Compared mutagenicity of EDC tar with EDC. The level of EDC present at the highest dose tested for EDC tar would only exert a weak mutagenic effect, yet a strong response was observed. 2. Activation of EDC tar dependent on NADPH. EDC activation independent of NADPH.

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TABLE 4-9. (continued)

Reference	Test system	Strain	Activation system	Chemical information	Results	Comments
Rannug and Ramel, 1977	Salmonella/ microsome assay (plate test)	TA1535	S9 mix from livers of uninduced male R strain Wistar rats plus NADPH-generating system	Concentration tested: Up to 45 umol/plate Source: BDH Chemicals, Ltd. Purity: Not given	Positive response (twofold increase) without activation; nearly ten-fold increase with activation. Negative controls yielded roughly 15 revertants/plate.	1. Compared mutagenicity of EDC tar with EDC. The level of EDC present at the highest dose tested for EDC tar would only exert a weak mutagenic effect, yet a strong response was observed. 2. Activation of EDC tar dependent on NADPH. EDC activation independent of NADPH.

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TABLE 4-9. (continued)

Reference	Test system	Strains	Activation system	Chemical information	Results	Comments
Rannug and Beije, 1979	Salmonella/ Body fluid analysis (isolated perfused rat liver)	TA1530 TA1535	Isolated perfused liver from male R strain Wistar rats	Concentration tested: 0.1 ml (1.3 mM) for up to 4 hours.	Positive. Highest response 15-60 min after addition of EDC (45-60 revertants compared to 7-10 in controls).	1. Positive responses consistent with conjugation of EDC with glutathione.
	Salmonella/ Body fluid analysis (bile)	TA1535	Bile from male CBA mice	80 mg/kg EDC i.p.; removal of liver and collection of bile 30 and 60 minutes later.	Positive. Greater than two-fold increases with bile from liver removed 30 min after addition of EDC (28.8 ± 2.7 revertants compared to 11.3 ± 1.1).	

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TABLE 4-9. (continued)

Reference	Test system	Strains	Activation system	Chemical information	Results	Comments
King et al., 1979	Salmonella/ microsome assay (plate test)	TA1535 TA100 TA1537 TA1538 TA98	PCB-induced rat liver S9	Concentration tested: 36 umol/plate	Negative	1. Standard plate incorporation test was conducted. No precautions were taken to prevent evaporation of EDC.
	E. coli K 12 strain 343/113 (suspension test and intra-sanguineous host-mediated assay)			10 mM (suspension assay)	Negative	
				2 mM/kg i.p. injection female NMRI mice	Negative	
				Source: Merck Co. Darmstadt, FRG		
				Purity: Not given. Stated that samples had correct melting point and elemental analysis		

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TABLE 4-9. (continued)

Reference	Test system	Strains	Activation system	Chemical information	Results	Comments
Nestmann et al., 1980	Salmonella/ microsome assay (plate test and desiccator exposure)	TA1535 TA100 TA1537 TA1538 TA98	PCB-induced rat liver S9 mix	Concentration tested: up to 9 mg/plate (91 umol) in desiccators. 10 mg/plate in plate tests. Source: Chem Service Purity: Not given Solvent: DMSO	Negative in standard test. Positive in desiccator test in strain TA1535.	1. Stated that maximum yield with TA100 is 20 revertants above background (i.e., negative). For TA1535 a doubling of mutant colonies per plate observed. No other data presented. 2. Cannot adequately evaluate results.

(continued on the following page)

TABLE 4-9. (continued)

Reference	Test system	Strains	Activation system	Chemical information	Results	Comments
Stolzenberg and Hine, 1980	Salmonella/ microsome assay (plate test)	TA100	PCB-induced rat liver S9 mix (2 mg protein/0.5 ml)	Concentration tested: Up to 10 umol/plate Source: Aldrich Chemical Co. Purity: 99% pure	Negative	1. All compounds tested in triplicate with and without S9 mix. 2. No precautions taken to prevent evaporation of test material. 2. Experimental values minus background revertants.

umol/plate	Revertants -S9	Revertants +S9
10^{-1}	0	0
1	0	0
10	15	No growth

(continued on the following page)

TABLE 4-9. (continued)

Reference	Test system	Strains	Activation system	Chemical information	Results	Comments
Barber et al., 1981	Salmonella/ microsome assay (vapor exposure)	TA1535 TA100 TA1538 TA98	PCB-induced rat liver S9 mix	Concentration tested: Up to 231.8 umol/plate as determined by GLC analysis of distilled water samples. Source: Eastman Kodak Purity: 99.98%	Negative in standard plate test in all strains. Positive in desiccator test in strains TA1535 and TA100.	1. Bacteria exposed in gastight exposure chambers. 2. Plastic plates found to absorb dibromomethane in parallel experiments. Thus, glass plates used for all other testing. 3. Weak positive result. 0.002 revertants/nmol in TA1535 with or without activation. 0.001 revertants/nmol TA100 with or without activation. 4. Revertants selected from each experiment and tested to ensure that they were actually his$^+$.

test agent. Positive and negative control tests were conducted for these experiments and indicated that the systems were working properly. Principe et al. (1981) conducted a spot test using Salmonella strains TA1535, TA1537, TA1538, TA98, and TA100. A positive response was observed for TA1535 when a triangular-shaped paper disc soaked with 100 ul EDC was placed on the agar in the presence of S9 from Aroclor 1254-induced rat liver (i.e., 103 revertants on the treated plate vs. 33 revertants for the negative control). Negative responses were obtained with all the other strains. A negative response was also obtained in plate incorporation tests conducted with strains TA100 and TA1535 at doses up to 100 ul/plate, but these tests involved no precautions to prevent excessive evaporation of the EDC. Similarly in forward mutation tests conducted with *Streptomyces coelicolor* and *Aspergillus nidulans*, negative responses were obtained at doses up to 100 and 500 ul/plate in plate incorporation and spot tests. There was a 100% survival in the test conducted with *S. coelicolor* and a 60% reduction in cell survival at the highest dose with *A. nidulans*. These tests provide less than adequate conditions for assessing the mutagenicity of EDC, because exposure to the test organisms may have been insufficient.

McCann et al. (1975) exposed Salmonella strains TA1535, TA100, and TA98 to EDC (Aldrich, stated to be highest purity available) concentrations as high as 13 mg/plate (131 umol/plate). A weak positive response was observed in TA100 (0.19 revertants/umol), but reproducible dose-response curves were not obtained. The presence of an exogenous S9 metabolic activation system (S9 mix from rat livers induced with either phenobarbital or Aroclor 1254) did not increase the weak positive response. Chloroacetic acid, which is a known mammalian metabolite of EDC, and the putative intermediates chloroethanol and chloroacetaldehyde were also tested for their mutagenic potential in strain TA100. Chloroacetic acid was negative, chloroethanol yielded a weak positive result, and chloroacetaldehyde a strong positive result (0.06 and 746 revertants/umol, respectively). McCann et al. (1975) The authors speculated that the weak mutagenic response of EDC may have been due to inefficient conversion of chloroethanol to chloroacetaldehyde.

Two reports (Rannug, 1976; Rannug and Ramel, 1977) compared the mutagenicity of EDC with that of EDC tar, a complex mixture formed during the manufacture of vinyl chloride from acetylene, ethylene, or a mixture of the two. The mixture is called EDC tar because EDC is a component, comprising about 30% of the mass. The sample of EDC tested in these studies was from British Drug House (BDH) Chemicals, Ltd. (purity not given). Salmonella tester strain TA1535 was treated with concentrations up to 45 umol/plate in the presence or absence of an exogenous metabolic activation system from livers of male strain R Wistar rats with and without NADP. Weak positive (almost twofold) increases in revertant frequencies over background (13.0 ± 1.76) were observed in the plates receiving the highest dose (24.8 ± 3.06) without metabolic activation. With activation, however, a stronger positive response was observed. The revertant count was elevated ninefold over the spontaneous level (132.8 ± 8.35 vs. 15.5 ± 1.21). The response to EDC was independent of the presence of NADP; in contrast, the mutagenicity of EDC tars was increased with metabolic activation only in the presence of NADP. The mutagenicity of EDC tar cannot be ascribed primarily to EDC, because EDC and EDC tar have different requirements for metabolic activation. In addition, the concentration of EDC present in EDC tar yields only a weak positive response at the highest dose if tested alone, and a strong response was observed for the EDC tar. In subsequent studies, Rannug et al. (1978) tested Salmonella strain TA1535 with EDC (BDH Chemicals Ltd.) at doses up to 60 umol/plate. Although the purity of EDC was not reported, the material was checked by glass capillary chromatography using a flame ionization detector. Tests were conducted with and without an exogenous rat liver metabolic activation system. The components of the activation system were varied to study the mechanism of activation of EDC. Phenobarbital-induced liver fractions from Sprague-Dawley or R strain Wistar rats were prepared with and without an NADPH-generating system and with and without glutathione transferases A, B, and C. Rannug et al. (1978) observed that EDC is activated by the cytosol fraction of liver homogenates; mixed-function oxygenases are not involved. The activation was NADPH-independent but required glutathione (GSH) A or C S-transferase enzyme activity. The extent of activation was dependent on the rat strain used (liver fractions from R strain rats were more effective than those from Sprague-Dawley rats) and the handling of the extract (e.g., storage on ice or freezing reduced the effectiveness of the activation system). The report by McCann et al. (1975) that EDC was not metabolized to a mutagenic intermediate may be due to differences in the exogenous activation system used (S9 mix rather than cytosol fraction). Rannug et al. (1978) hypothesized that the mutagenicity of EDC after metabolic activation was caused by the formation of a highly reactive half sulfur mustard, S-(2-chloroethyl)-L-cysteine. This compound (>99% purity) was found to be more strongly mutagenic in TA1535 than EDC. At 0.2 umol/plate, 12.8 ± 1.5 and 176.8 ± 11.1 revertants were observed per plate after treatment with EDC and S(2-chloroethyl)-L-cysteine, respectively. At 5.0 umol/plate, the observed numbers of revertants per plate were 13.0 ± 1.1 and 1945 (only one plate tested), respectively. Thus, S-(2-chloroethyl)-L-cysteine gives a strong direct mutagenic effect in strain TA1535 at low doses at which no effect is seen for EDC.

In intact mammals most GSH conjugates are normally excreted in bile. Rannug and Bieje (1979) reasoned that if EDC were metabolically activated by conjugation with glutathione to form a half sulfur mustard, mutagenic products would be found in the bile of EDC-treated mammals or perfused livers. To test this hypothesis, an EDC concentration of 0.1 ml (1.3 mmol) was perfused through R strain Wistar rat livers for up to 4 hours. Bile was collected immediately after addition of EDC and 0.25, 0.5, 1, 1.5, 1.75, 2, 3, and 4 hours later. Bile was tested directly or diluted 5 to 10 times in sterile water and added to top agar containing Salmonella strain TA1535 for plate incorporation tests. Positive responses were obtained. The greatest response (45-60 revertants per plate, compared with 7-10 in negative controls) was reached between 15 minutes and 1 hour after addition of EDC. In a second experiment, 80 mg/kg EDC was given to male CBA mice intraperitoneally. The animals were sacrificed, their livers removed, and bile collected 30 and 60 minutes later for mutagenicity testing with strain TA1535 in plate incorporation tests. An increase in revertants (greater than twofold) was observed for bile collected 30 minutes after treatment compared with bile from negative control animals (28.8 ± 2.7 revertants and 11.3 ± 1.1 revertants, respectively). The positive responses obtained with bile from the perfused rat livers and intact mouse livers are consistent with the hypothesis that EDC is activated by conjugation with glutathione.

Four studies have been reported in which EDC was found to be negative in the standard Salmonella/microsome assay plate incorporation test (King et al., 1979; Nestmann et al., 1980; Stolzenberg and Hine, 1980; Barber et al., 1981). The maximum doses employed in the first three studies were 36 umol/plate, 91 umol/plate, and 10 umol/plate, respectively. The doses in these studies are in the range in which positive responses have been reported in other studies.

The fourth study did not report the doses used. Each test was conducted with and without PCB-induced rat liver S9 mix. Except for the report by Stolzenberg and Hine (1980), the negative Salmonella plate incorporation studies were conducted with appropriate positive controls. The positive controls that require activation, however, were not structurally similar to EDC. These positive controls may not be able to determine the effectiveness of the components in the S9 mix necessary for EDC activation. King et al. (1979) also reported negative responses when E. coli K12 strain 343/113 was tested in either a liquid suspension test or an intrasanguineous host-mediated assay.

Two of the studies (Nestmann et al., 1980; Barber et al., 1981) reported that EDC did not induce mutations in standard plate incorporation tests, but did cause positive responses when the studies were conducted in airtight exposure chambers. Nestmann et al. (1980) exposed Salmonella strains TA1535 and TA100 to doses from 3 to 9 mg/plate (30 to 91 umol/plate) in desiccators. It was reported that this treatment yielded positive results, at least for TA1535, in which there was a doubling in the number of mutant colonies over the control. It was stated that concentrations were tested up to levels where cell killing was observed, but no data are given and insufficient detail is provided to allow the results to be independently evaluated.

Barber et al. (1981) exposed Salmonella tester strains TA1535, TA98, and TA100 to four levels of EDC vapors (Eastman Kodak Co., 99.98% pure) in a 3.4 L airtight exposure chamber. These exposures resulted in estimated plate concentrations ranging from 31.8 to 231.8 umol/plate as determined by gas liquid chromatography analysis of distilled water samples placed in the exposure chamber. Linear, dose-related increases in revertant counts were observed for TA1535 and TA100. The mutagenicity of EDC in these two strains was 0.002 and

0.001 revertants/nmol, respectively. No difference in revertant counts or potency of EDC was noted in comparisons of tests done with and without metabolic activation. The positive results were obtained by Nestmann et al. (1980) and Barber et al. (1981) only when tests were conducted in airtight exposure chambers. This suggests that standard mutagenicity testing of EDC (bp 83° - 84°C) may not provide an adequate assessment of its mutagenic potential due to excessive evaporation.

In summary, positive responses obtained without metabolic activation indicate that EDC is a weak direct-acting mutagen in bacteria. Positive responses obtained with metabolic activation indicate that one or more of its metabolites are more potent mutagens. The negative findings reported in some bacterial tests are not considered to contradict the reported positive results, because the negative results may have been due to evaporation of EDC or to inadequacy of the S9 activation system.

4.4.1.2. Higher Plants--Two reports on the ability of EDC to induce mutations in higher plants are summarized in Table 4-10. Ehrenberg et al. (1974) treated barley seeds (variety Bonus) with 30.3 mmol EDC (Merck, purity not given) for 24 hours and scored for sterile spikelets at maturity or chlorophyll mutations in about 600 spike progenies in the subsequent generation. The dose corresponds to the LD_{50}. EDC-treated kernels had an increased incidence of chlorophyll mutations (6.8%) compared with untreated controls (0.06%).

Kirichek (1974) exposed eight varieties of pea seeds (100 each) to gaseous EDC (purity and concentration not given) for 4 hours. Germination of treated seeds differed with the variety tested but was reduced (15-50% germination) compared with negative control seeds exposed to water vapor for 4 hours (100% germination). From 5.4 to 28.13% of the seeds were reported to be mutated.

TABLE 4-10. SUMMARY OF MUTAGENICITY TESTING OF EDC: HIGHER PLANTS

Reference	Test system	Chemical information	Results	Comments
Ehrenberg et al., 1974	Segregating chlorophyll (gene) mutations in barley	Concentration tested: 200 seeds treated with 30.3 mmol/24 hours (LD_{50}) in a closed vessel.	Positive response. (6-8% mutants from treated progeny vs. 0.06% mutants from control progeny). Source: Merck Co. Darmstadt, FRG Purity: Not given	1. About 600 spike progeny were tested.
Kirichek, 1974	Morphological mutations in peas, 8 varieties	Concentration tested: EDC (concentration not reported) or water (negative controls) vapors for 4 hours. Source: Not reported Purity: Not reported	Reported positive. 5.42-28.13% of seeds reported to be mutated.	1. English translation of Russian article. 2. Germination of the treated seeds varied with the variety tested from 15% to 58% compared to 100% germination of the control seeds. 3. Control mutation freqency not given. 4. Putative mutations not characterized. 5. Not possible to evaluate results adequately.

Kirichek (1974) reported that two times as many morphological mutants were induced as chlorophyll mutations and considered this a positive response, but the mutation frequency for negative controls was not given. This limitation of the report plus a lack of information concerning the characterization of the putative mutations precludes an adequate evaluation of the results.

4.4.1.3. Insects--Four studies on the ability of EDC to cause mutations in Drosophila melanogaster are summarized in Table 4-11. Three studies demonstrated the ability of EDC to cause sex-linked recessive lethal mutations (Shakarnis, 1969; Shakarnis, 1970; King et al., 1979). The fourth study demonstrated the induction of somatic cell mutations by EDC (Nylander et al., 1979).

Shakarnis (1969, 1970) performed two experiments to assess the ability of EDC (purity not given) to cause sex-linked recessive lethal mutations in Drosophila. In the first study (Shakarnis, 1969), adult females from a radiosensitive strain (Canton-S) were exposed to 0.07% EDC gas for 4 or 8 hours at 24° - 25°C. Immediately after treatment, the females were mated to Basc males. The fertility of treated females was reduced 47% by the 4 hours treatment and 91% by the 8-hour treatment. The F_2 progeny were scored for lethality (measured as the absence of Basc males). A statistically significant ($P < 0.05$) time-related increase in the incidence of sex-linked recessive lethals was observed. The frequencies of lethals were 0.3% (negative controls), 3.2% (4-hour treatment) and 5.9% (8 h treatment). In his second study, Shakarnis (1970) exposed females from a radiostable strain (D-32) of D. melanogaster to 0.07% EDC vapors (source and purity not reported) for 4 or 6 hours. Immediately after treatment they were mated to Basc 5 males and the F_2 progeny scored for sex-linked recessive lethals. The 4-hour treatment did not significantly reduce fertility, but the 6-hour treatment reduced it by 50%. As in the first study, a

TABLE 4-11. SUMMARY OF MUTAGENICITY TESTING OF EDC: GENE MUTATION TESTS IN INSECTS

Reference	Test system	Chemical information	Results	Comments
Shakarnis, 1969	*Drosophila melanogaster* sex-linked recessive lethal test	Concentration tested: virgin 3-day-old Canton S females exposed to 0.07% EDC gas for 4 or 8 h at 24-25°C. Source: Not given Purity: Not given	Positive response	1. Basc males. 2. Fertility of treated females reduced significantly (47% reduction after 4-h treatment and 91% after 8-h treatment).

Duration of treatment (h) with 0.07% EDC	Number of Chromosomes Scored	Lethal Mutations No.	Lethal Mutations %
Control	4910	16	0.32
4	2081	67	3.22
8	4750	281	5.91 $P < 0.05$

(continued on the following page)

TABLE 4-11. (continued)

Reference	Test system	Chemical information	Results	Comments
Shakarnis, 1970	*Drosophila melanogaster* sex-linked recessive lethal test	Concentration tested: virgin 3-day-old D-32 strain females exposed to 0.07% EDC gas for 4 or 6 h at 24-25°C. Source: Not given Purity: Not given	Positive response	1. Basc males. 2. Experiment conducted twice for 4-h exposure and three times for 6-h exposure. 3. 4-h treatment did not significantly reduce fertility but 6-h treatment reduced it by 50%.

Duration of treatment (h) with 0.07% EDC	Number of chromosomes scored	Lethal mutations No.	Lethal mutations %
Control	1904	1	0.05
4	2205	46	2.00
6	2362	78	3.30 ($P < 0.05$)

(continued on the following page)

TABLE 4-11. (continued)

Reference	Test system	Chemical information	Results	Comments
King et al., 1979	*Drosophila melanogaster* sex-linked recessive lethal test	Concentration tested: 50 mM solution of EDC in 5% sucrose fed to 1- to 2-day-old Berlin-K males for 3 days (near LD_{50}). Source: Merck Co. Darmstadt, FRG Purity: Not given but stated to have correct melting point and elemental analysis.	Positive response	1. Basc females. 2. Lethal frequency increased for all broods. Greatest effect in brood II, which corresponds to the spermatid stage.

Conc. (mM)	Brood	Days after treatment	Number of chromosomes scored	Lethal mutations No.	Lethal mutations %	Lethal mutations P value
0	I-III	0-9	22,048	47	0.21	
50	I	0-3	1,185	6	0.51	
	II	4-6	1,179	41	3.48	<0.01
	III	7-9	156	2	1.28	
	I-III	0-9	2,520	49	1.94	<0.01

(continued on the following page)

TABLE 4-11. (continued)

Reference	Test system	Chemical information	Results	Comments
Nylander et al., 1979	*Drosophila melanogaster* somatic cell mutations sc z w^+ and z Dpw^+61e19 stocks.	Concentration tested: 0.01% and 0.5% EDC in food at 25°C and 75% humidity during larval development Source: Fisher Scientific Co. Purity: Not given	Positive response	1. Mutations at z locus scored in flies of stable and unstable genotype. Both have same phenotype. Instability thought to be caused by transposable genetic element. 2. Survival of treated flies reduced significantly in both genetically stable (34% reduction for 0.1% EDC and 77% reduction for 0.5% EDC) and genetically unstable stocks (86% reduction for 0.5% EDC).

Treatment	Number males scored	Number with sectors	%	t value differences within genotypes		t value differences between genotypes	
Control							
Stable	4441	2	0.045	---		0.60	
Unstable	5363	4	0.075	---			
0.01%							
Stable	6260	263	4.20	18.74^a	$p < 0.001$		
Unstable	2889	274	9.28	24.31^a	$p < 0.001$	9.16^a	$p < 0.001$
0.05%							
Stable	610	44	7.21	11.46^a	$p < 0.001$		
Unstable	201	50	24.88	13.12^a	$p < 0.001$	5.66^a	$p < 0.001$

statistically significant ($P < 0.05$) time-related increase in sex-linked recessive lethals was observed. A frequency of 0.05% lethals was observed in the negative controls, and frequencies of 2 and 3.30% lethals were observed in offspring from the 4-hour and 6-hour treatments, respectively.

King et al. (1979) also studied the ability of EDC to induce sex-linked recessive lethals in Drosophila. One- to two-day-old Berlin-K males were fed a 50 mM solution of EDC (Merck and Co., purity not given but correct melting point [sic] and elemental analysis reported) in 5% sucrose for 3 days. This dose approximated the LD_{50}. The males were mated to virgin Basc females every 3 days for a total of 9 days to determine if EDC preferentially damaged particular stages in spermatogenesis. Progeny of the F_2 generation were scored for lethality. The lethal frequency was found to be elevated for all three broods (0.51%, 3.48%, and 1.28% for broods, I, II, and III, respectively). compared with the negative controls (0.21%). The highest lethal frequency (3.48%) was found in brood II ($P < 0.01$), which corresponds primarily to the spermatid stage of spermatogenesis.

Nylander et al. (1978, 1979) raised flies on food containing 0.1 and 0.5% EDC (Fisher Scientific Co., purity not given) during larval development and scored F_1 progeny for the induction of somatic cell sex-linked mutations in the eye. A genetically unstable stock ($\underline{sc}\ \underline{z}\ \underline{w}^+$) and a genetically stable stock ($\underline{z}\ \underline{Dp}\ \underline{w^{+61e19}}$) both having the same phenotype were used in these studies and mated to attached X females. Mutations result in the expression of normal (dark red) eye pigment sectors in emerging adult treated males. The genetic instability of $\underline{sc}\ \underline{z}\ \underline{w}^+$ is caused by the insertion of a transposable genetic element. The survival of flies raised on the EDC-treated food was reduced compared with the negative control values (e.g., 77% reduction for the stable stock and 86%

reduction of the unstable stock at 0.5% EDC). Statistically significant increases in somatic cell mutations were observed in both stocks at both the low and high doses ($P < 0.001$).

The above positive responses in Drosophila indicate that EDC is capable of causing both somatic cell and heritable germinal mutations in a multicellular eucaryote.

4.4.1.4. Mammalian Cells in Culture--Tan and Hsie (1981) found that EDC (Matheson, Coleman and Bell, purity not given) causes a dose-related increase in HGPRT mutations in cultured Chinese hamster ovary (CHO) cells (Table 4-12). CHO-K_1-BH_4 cells were exposed in suspension culture for 5 hours to EDC concentrations ranging up to 3 mM (30% survival) in tests with exogenous rat liver S9 mix and concentrations ranging up to 50 mM (50% survival) in tests conducted without metabolic activation. Weak positive responses were observed both in tests with and without metabolic activation. EDC was detected as a direct-acting mutagen only at high doses, but the induced mutation frequency was increased about tenfold over control values. About a fourfold additional increase in mutagenicity was noted with metabolic activation, but toxicity precluded testing at concentrations greater than 3 mM. The metabolic activation system was only effective in the presence of NADP; this result differs from that of Rannug (1976) who found metabolic activation of EDC to be NADP-independent.

4.4.1.5. Whole Mammals--Gocke et al. (1983) studied the ability of EDC to cause somatic cell mutations in the mouse spot test (Table 4-13). This test was a follow-up to an earlier study (King et al., 1979) of EDC in the Salmonella/microsome assay, Drosophila sex-linked recessive lethal test, and mouse micronucleus test. One intraperitoneal injection of EDC (from Merck, Darmstadt, FRG; purity not reported) at 300 mg/kg was administered in olive

TABLE 4-12. SUMMARY OF MUTAGENICITY TESTING OF EDC: MAMMALIAN CELLS IN CULTURE

Reference	Test system	Chemical information	Results	Comments
Tan and Hsie, 1981	Chinese hamster ovary cell HGPRT gene mutation assay	Concentration tested: Up to 3 mM in tests with rat liver S9 mix and up to 50 mM in tests without metabolic activation. LD_{50} is 1 mM with activation and 6 mM without activation.	Positive response: 60 vs. 3 mutants/10^6 clonable cells for 50 and 0 mM EDC without activation; 28 vs. 3 mutants/10^6 clonable cells for 1.5 and 0 mM EDC with activation.	1. Mutagenic activity of EDC without and with metabolic activation calculated to be 1 and 5 mutants/10^6 cells/mM respectively. 2. S9 mix increases mutagenicity by about fourfold and cytotoxicity 5- to 25-fold. 3. NADP required in S9 mix for metabolic activation.

oil to 525 C57Bl females on the 10th day of pregnancy after mating with T-stock males. There were 1,104 progeny examined; 49% of the females gave birth and the average litter size at birth was 4.9 and at weaning was 4.3. Seven of the offspring had spots indicative of somatic cell mutagenicity (0.6%) compared with 2/812 in the olive oil controls (0.3%) and 3/2,161 for all the controls (0.1%). Analyses of these data by the Fisher exact test showed a signficant (P = 0.03) effect against the cumulative control, but no significance (P = 0.18) against the olive oil control. The data suggest a possible weak mutagenic effect of EDC in the mouse. With the control values reported in this study, testing more than 10,000 animals would be necessary to secure a doubling of the spontaneous relevant spot frequency.

The consistency of positive results obtained in higher plants, Drosophila, and cultured mammalian cells and the suggestive evidence for mutagenicity in the mouse spot test is sufficient to demonstrate that EDC causes gene mutations in eucaryotes.

4.4.2. Cytogenetic Studies

Five studies were evaluated on the ability of EDC to cause chromosomal damage (Table 4-14). One was an abstract on chromosome breakage and C-mitosis in Allium root tips and cultured human lymphocytes (Kristoffersson, 1974). Two were *D. melanogaster* sex chromosome nondisjunction tests (Shakarnis, 1969, 1970), and two were micronucleus tests (King et al., 1979; Jenssen and Ramsel, 1980).

In the abstract by Kristoffersson (1974) EDC (source, purity, and concentration not given) was reported to produce C-mitosis in Allium root tip cells but not chromosome breaks in Allium root tip cells or human lymphocytes or to induce prophage from *E. coli* K 39 (λ). Insufficient information, however, was available

TABLE 4-13. SUMMARY OF MUTAGENICITY TESTING OF EDC: MOUSE SPOT TEST

Reference	Test system	Chemical information	Results	Comments
Gocke et al., 1983	C57Bl female X T-stock male	One i.p. injection of 300 mg/kg EDC in olive oil 10 days after conception. Source: Merck Co. Darmstadt, FRG Purity: Not given	7/1,104 offspring had spots compared with 2/812 in olive oil controls and 3/2,161 for 211 the cumulative controls.	Data suggestive of a positive response.

TABLE 4-14. SUMMARY OF MUTAGENICITY TESTING OF EDC: "CHROMOSOMAL DAMAGE TESTS"

Reference	Test system	Chemical information	Results	Comments
Shakarnis, 1969	Drosophila melanogaster X chromosome nondisjunction	Concentration tested: virgin Canton-S females exposed to 0.7% EDC in 1.5 liter desiccator for 4 or 8 h at 24-25°C. Source: Not given Purity: Not given	Statistically significant ($P < 0.05$) increases in exceptional female progeny for 4 h exposure and male and female progeny for 8 h exposure.	

0.07% EDC duration of treatment (h)	Normal progeny No. females	Normal progeny No. males	Exceptional females No.	Exceptional females %	Progeny males No.	Progeny males %
Control	5,848	5,727	0	0[a]	0	0[b]
4	24,125	21,297	8	0.03 ($p<<0.05$)	3	0.01
8	8,437	7,773	15	0.18 ($p<<0.05$)	7	0.09 ($p<<0.05$)

(continued on the following page)

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[a] Based on female progeny only.

[b] Based on male progeny only.

TABLE 4-14. (continued)

Reference	Test system	Chemical information	Results	Comments
Shakarnis, 1970	*Drosophila melanogaster* X chromosome nondisjunction	Concentration tested: 3-day-old females from radiostable D-32 strain exposed to 0.07% EDC in a 1.5 L desiccator for 4 or 6 h at 24-25°C. Source: Not given Purity: Not given	Incidence of non-disjunction greater in treated group than in control. Increase not statistically significant.	1. Not as many individuals scored as in 1969 study. Sample size may not have been sufficiently large. 2. Longest exposure times 2 h shorter than in previous experiment; may have been too short.

0.07% EDC duration of treatment (h)	Normal progeny		Exceptional females		Progeny males	
	No. females	No. males	No.	%	No.	%
Control	2472	2205	0	0	1	0.03
4	3584	3401	1	0.03	1	0.03
6	4043	4090	4	0.10	3	0.07

(continued on the following page)

TABLE 4-14. (continued)

Reference	Test system	Chemical information	Results	Comments
King et al., 1979	Micronucleus test: NMRI mice	Concentration tested: 2 i.p. injections of 4 mmol/kg (400 mg/kg) given 24 h apart; 4 animals/dose sacrificed after second injection. Source: Merck Co. Darmstadt, FRG Purity: Not specified, but melting point and elemental analysis were performed	Negative	1. 1,000 polychromatic erythrocytes analyzed/animal. 2. Frequency of micronuclei not given.
Jenssen and Ramel, 1980	Micronucleus test: CBA mice	Concentration tested: single i.p. injection at 100 mg/kg. Source: BDH Chemicals, Ltd. Purity: Not given	Negative. (0.15 ± 0.14) polychromatic erythrocytes with micronuclei in controls vs. 0.17 ± 0.10 in treated animals).	

to substantiate the reported results.

In studies by Shakarnis (1969, 1970) using D. melanogaster, females were exposed to 0.07% EDC vapors (source and purity not given). In the first study (1969) a statistically significant ($P < 0.05$) increase in exceptional F_1 progeny, indicative of meiotic nondisjunction, was seen after a 4 h treatment (0.03% exceptional females) and an 8-hour treatment (0.18% exceptional females and 0.09% exceptional males) compared with untreated controls (0%; 0/11,575 progeny). However, not only was the longest treatment time some 25% shorter than in the first study (6 hours vs. 8 hours) but a sample size at least two-to-threefold as large was required to confirm the data as statistically significant for the observed differences in rates between the control and treated series. Accordingly, the second study is of considerably less value in the assessment of the mutagenic potential of the compound.

Micronucleus tests were performed by King et al. (1979) and Jenssen and Ramel (1980). Both studies reported negative results. King et al. (1979) gave NMRI mice two intraperitoneal injections of 4 mmoles/kg (400 mg/kg) EDC (Merck Co., purity not specified but melting point [sic] and chemical analysis reported to be correct). King et al. (1979) state this treatment corresponds to an "approximate lethal dose" (the LD_{10} for intraperitoneal injections of EDC in mice is 250 mg/kg). The injections were given 24 hours apart, and the animals were killed 6 hours after the second injection. Bone marrow smears were made; one thousand polychromatic erythrocytes (PCEs) were analyzed per animal. Frequencies of micronuclei were not given, but the results were regarded by King et al. as negative.

Jenssen and Ramel (1980) also reported negative results in a micronucleus test. CBA mice were given a single intraperitoneal injection of EDC (BDH

Chemicals Ltd., England, purity not given) of 100 mg/kg. The animals were sacrificed the next day and PCEs (number not given) were scored for micronuclei. The frequencies of PCEs with micronuclei were 0.15 ± 0.14 in the controls and 0.17 ± 0.10 in treated animals.

The positive response in the X-chromosome loss test in Drosophila (Shakarnis, 1969) suggests EDC is capable of causing meiotic nondisjunction resulting in numerical chromosomal abnormalities. The negative responses obtained in the micronucleus tests may indicate that EDC does not cause chromosomal damage in mice. However, because EDC has not been adequately tested for its ability to cause structural chromosomal aberrations, it would be appropriate to perform mammalian in vitro and in vivo cytogenetic tests. Such testing is required before a judgment can be made on the ability of EDC to cause chromosomal aberrations.

4.4.3. Other Studies Indicative of DNA Damage

Three other tests have been conducted on the genotoxicity of EDC. These tests do not measure mutagenic events per se in that they do not demonstrate the induction of heritable (i.e., somatic or germinal) genetic alterations, but positive results in these test systems are taken as evidence that DNA has been damaged. Such test systems provide supporting evidence that is useful for assessing genetic risk.

4.4.3.1. Bacteria--EDC is reported to be positive in the polA assay, which measures toxicity associated with unrepaired damage in DNA (Table 4-15). Brem et al. (1974) soaked sterile filter disks with 10 ul (80 umol) EDC. The disks were centered on the agar surface of petri dishes of bacteria. One set of plates contained a $polA^+$ strain, while the other contained a $polA^-$ strain. After incubation the plates were scored for differential inhibition of growth. An 8-mm zone of growth inhibition was observed in the $polA^+$ strain compared

TABLE 4-15. SUMMARY OF MUTAGENICITY TESTING OF EDC: polA ASSAY

Reference	Test system	Strains	Activation system	Chemical information	Results	Comments
Brem et al., 1974	polA differential cell killing assay	*E. coli* polA$^-$ polA$^+$	None	Concentration tested: 10 ul on filter disk	Questionable positive	1. All assays carried out in duplicate on at least three different occasions. 2. Only a small difference in diameter (i.e., 1 mm) was noted between the zones of killing in the two strains. Thus, the A$^+$/A$^-$ ratio may be misleading.

	polA zone of inhib.		
	polA$^+$ mm	polA$^-$ mm	polA$^+$/polA$^-$
EDC	8	9	1.26
MMS	45	54	1.44
Chloramphenicol	28	28	1.00

with a 9-mm zone of inhibition in the polA$^-$ strain. These responses were reported to be reproducible. The ratio between the zones of inhibition (polA$^+$/polA$^-$) was 1.26, which is interpreted by Brem et al. as a positive response. Since 1 mm is not a big difference, however, the result could also be regarded as equivocal.

4.4.3.2. Eucaryotes--One test has been performed to assess the ability of EDC to cause UDS (Perocco and Prodi, 1981). Perocco and Prodi (1981) collected blood samples from healthy humans, separated the lymphocytes, and cultured 5 x 10^5 in 0.2 ml medium for 4 hours at 37°C in the presence or absence of EDC (Carlo Erban, Milan, Italy or Merck-Schuchardt, Darmstadt, FRG, 97-99% pure). The tests were conducted both in the presence and in the absence of PCB-induced rat liver S9 mix. A comparison was made between treated and untreated cells for scheduled DNA synthesis (i.e., DNA semi-conservative replication) and UDS. No difference was noted between the groups with respect to scheduled DNA synthesis measured as dpm of [^{3}H] deoxythymidylic acid (TdR) after 4 hours of culture (2,661 ± 57 dpm in untreated cells compared to 2,287 ± 60 dpm in cells treated with 5 ul/ml [0.06 umol/ml] EDC). Subsequently 2.5, 5, and 10 ul/ml (0.03, 0.06, and 0.1 umol/ml) EDC was added to cells followed by 10 mM hydroxyurea to suppress scheduled DNA synthesis. The amount of unscheduled DNA synthesis was estimated by measuring dpm from incorporated [^{3}H]TdR 4 hours later. At 10 ul/ml EDC, 483 ± 37 and 532 ± 21 dpm were counted without and with exogenous metabolic activation, respectively. Both values were lower than corresponding negative controls of 715 ± 24 and 612 ± 26 dpm, respectively. No positive controls were run to ensure that the system was working properly, although testing of chloromethyl methyl ether (CMME) with activation resulted in a doubling of dpms over the corresponding negative control values (1,320 ± 57 at 5 ul/ml CMME vs.

612 ± 26 untreated). Perocco and Prodi (1981) calculated an effective DNA repair value (r) for each chemical based on the control and experimental values with and without metabolic activation. Ethylene dichloride was evaluated by the authors as positive in the test, but they did not state their criteria for a positive. None of the experimental values from cells treated with EDC without metabolic activation had higher dpm values than the controls. Although two out of three experimental values were greater than the controls with metabolic activation (673 ± 45 at 5 ul/ml and 630 ± 34 at 2.5 ul/ml compared with control value of 612 ± 26), the increases were not statistically significant. The results reported in this work are inconclusive.

4.4.4. DNA Alkylation Studies

Reitz et al. (1982) compared the pharmacokinetics of EDC administered to Osborne-Mendel rats by inhalation and by gavage. DNA alkylation was also measured in bacteria at EDC cytosol concentrations corresponding to those used in the DNA binding study (Table 4-16). Two gram aliquots of TA1535 were incubated with 7.06 umol [^{14}C] EDC/ml (sp. act. = 3.2 mCi/mmol) and varying amounts of cytosol. DNA alkylation values at cytosol concentrations of 2.2, 7.8, 27, and 71% were 8.65, 27, 107, and 137 dpm/mg purified DNA, respectively. This corresponds to 4, 12.5, 49, and 64 alkylations x 10^{-6} DNA nucleotides. The corresponding reversion frequencies were 4.6 ± 0.82, 23.5 ± 3.0, 80.2 ± 9.6, and 111 ± 2.6, respectively (n = 3 in each case). A direct correlation between the degree of alkylation and an increase in mutation frequency was indicated by linear regression analysis (r = 0.9976). In the DNA alkylation studies with rats [^{14}C] EDC (sp. act. = 0.32 mCi/mmol) was administered to groups of three animals by gavage (150 mg/kg) or inhalation (150 ppm, 6 hours). The animals were sacrificed and DNA was extracted from the liver, spleen, kidney, and stomach for measurement

TABLE 4-16. SUMMARY OF MUTAGENICITY TESTING OF EDC: DNA BINDING STUDIES

Reference	Test system	Strains	Activation system	Chemical information			Comments
Reitz et al., 1982	DNA alkylation and mutagenesis in Salmonella	TA1535	Phenobarbital-induced rat liver cytosol	% Cytosol	Revertants	Alkylation x 10^{-6} Nucleotides	1. Bacteria incubated with 7.06 umol EDC/ml.
				2.2	4.6 ± 0.82	4	2. Significant correlation between degree of alkylation and increased reversion frequency (r = 0.9976).
				7.8	23.5 ± 3.0	12.5	
				27	80.2 ± 9.6	49	
				71	111 ± 2.6	64	
	DNA alkylation in rats	Osborne-Mendel rat	NA	Route of Exposure	Tissue	Alkylation x 10^{-6} Nucleotides (means from 2 experiments)	
				Gavage 150 mg/kg	Liver	21.3 ; 13.9	3. Rats sacrificed 4 h post gavage or immediately after inhalation.
					Spleen	5.8 ; 2.5	
					Kidney	17.4 ; 14.5	
					Stomach	14.9 ; 6.7	
				Inhalation 150 ppm, 6 hours	Liver	8.2 ; 3.3	4. Specific activity 3.2 mCi/mmol for bacteria; 0.32 mCi/mmol for rats
					Spleen	1.8 ; 1.8	
					Kidney	5.2 ; 2.0	
					Stomach	2.8 ; 1.9	

of DNA alkylation. Overall, three to five times more DNA alkylation was detected after gavage than after inhalation. The values ranged from 5.8 ± 0.7 to 23.1 ± 7.4 alkylation/10^6 nucleotides for gavage vs. 1.8 ± 0.3 to 8.2 ± 3.3 alkylation/10^6 nucleotides for inhalation. Under the conditions of the test (Reitz et al., 1982), EDC exposure resulted in a similar degree of adduct formation in rats and bacteria. Because DNA is the genetic material in both bacteria and rats, these data predict that mutations were induced in the rat at the exposures used. This prediction is in keeping with the positive results in mutagenicity tests in other eucaryotes including Drosophila and cultured CHO cells. DNA alkylation was not measured in rat gonads, so it is not possible to estimate the heritable genetic risk.

4.4.5. Summary and Conclusions

Ethylene dichloride (EDC) has been shown to induce gene mutations in bacteria, plants, Drosophila, and cultured Chinese hamster ovary (CHO) cells. Weak positive responses were observed in the bacterial and CHO tests in the absence of an exogenous metabolic activation system. Stronger positive responses were found when hepatic metabolic activation systems were incorporated. Based on these positive findings in different test systems representing a wide range of organisms, EDC is capable of causing gene mutations.

EDC has been reported to cause meiotic chromosomal nondisjunction in Drosophila. The induction of meiotic nondisjunction is a significant genotoxic effect; however, a positive response in another test system is needed to permit a judgment on the generality of this effect. With respect to its ability to cause structural chromosomal aberrations, sufficient testing has not been performed. There is only one abstract published on the ability of EDC to cause structural chromosomal aberrations (i.e., in Allium root tip cells and human lymphocytes). Because no data were presented, an independent assessment of the author's conclusions is not possible. Even though negative results are reported from micronucleus tests, additional information is needed to draw conclusions on the ability of EDC to cause chromosomal aberrations. For example, it would be appropriate to test EDC in in vitro and in vivo mammalian cytogenetic assays including a sister chromatid exchange assay.

There are no available data on the ability of EDC to damage DNA in mammalian germ cells. Studies on the ability of EDC to reach germinal tissue would be appropriate to determine whether EDC has the potential to cause heritable mutations which may contribute to genetic disease. The finding that EDC causes heritable mutations in Drosophila and alkylates DNA in several somatic tissues in the rat reinforces the need for germ-cell studies in mammals.

Based on the weight of evidence, EDC is a weak direct-acting mutagen. Several of its putative metabolites, thought to be formed in mammals, are more potent mutagens (e.g., S-[2-chloroethyl]-L-cysteine or chloroacetaldehyde) than EDC. Such metabolites have the potential of causing adverse effects in humans. The array of damage produced by EDC is similar to that of EDB. In keeping with the relative electrophilicities of the chlorine and bromine atoms, EDB is a better alkylating agent and more potent mutagen.

4.5. METHYL BROMIDE (BROMOMETHANE)

Methyl bromide is a gaseous compound that has been evaluated for mutagenic activity in bacteria and in eucaryotes (Drosophila, mammalian cells in culture, intact rodents). The ability of methyl bromide to alkylate DNA has been determined in liver and spleen tissue from the mouse. The available studies are discussed below and summarized in Table 4-17.

4.5.1. Gene Mutation Studies

4.5.1.1. Bacteria--Methyl bromide has been tested for mutagenicity in the Salmonella/microsome test for reverse mutation and in *E. coli* and *Klebsiella pneumoniae* for forward mutation. Because methyl bromide is a gaseous compound (the boiling point is 3.56°C, vapor pressure at 20°C is 1420 mm Hg), studies were generally conducted in sealed containers or desiccators. Some liquid suspension studies were also performed. Methyl bromide was detected as mutagenic in all bacterial tests. Metabolic activation was not required. In those cases where S9 activation was used, the mutagenic responses were not enhanced (or decreased).

In Salmonella tests, methyl bromide was active in base-pair substitution strains while ineffective in frameshift-sensitive strains. Most studies, however, used tester strain TA100, which responds both to base-pair substitution mutagens and frameshift mutagens.

Moriya et al. (1983) evaluated 228 pesticides, including methyl bromide (purity not reported)*, in Salmonella tester strains TA98, TA100, TA1535, TA1538, and TA1537 and in *E. coli WP2 hcr*. Methyl bromide was injected into a sealed container from a syringe and incubated at 37°C for 2 days with constant stirring

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*Most pesticide samples were obtained from the Agricultural Chemicals Inspection Station of the Ministry of Agriculture, Forestry and Fisheries in Japan (Kodaira).

TABLE 4-17. SUMMMARY OF MUTAGENICITY TESTING OF METHYL BROMIDE

Test system	Reported results	Comment	Reference
A. Gene Mutation			
Bacteria:			
Salmonella/microsome assay	+	dose-response; active in base-pair substitution strains (TA100, TA1535); no enhanced response with S9 activation, tested in gaseous phase or liquid suspension	Moriya et al., 1983 Simmon, 1981; Kramers et al., unpublished; Voogd et al., 1982 (Abstract)
Escherichia coli WP2 reverse mutation assay and *Sd-4* forward mutation assay	+	dose-related response without S9	Moriya et al., 1983 Djalali-Behzad, 1981
Klebsiella pneumoniae	+	dose-related response without S9	Kramers et al., unpublished; Voogd et al., 1982; (Abstract)
Eucaryotes:			
L5178Y mouse lymphoma cell forward mutation assay	+	dose-related response without S9	Kramers et al., unpublished; Voogd et al., 1982, (Abstract)
Drosophila sex-linked recessive lethal test	+	1.25% total lethals is highest frequency observed	Kramers et al., unpublished; Kramers and Bissumbhar, 1983 (Abstract); Voogd et al., 1982, (Abstract)
	-	nontoxic doses tested, small sample size, no negative control data, results considered inconclusive	McGregor, 1981

(continued on the following page)

TABLE 4-17. (continued)

Test system	Reported results	Comment	Reference
B. Chromosome Aberrations			
Rat bone marrow assay	-	dosage levels may have been too low to preclude weak activity, gaps observed in males only	McGregor, 1981
Rat dominant lethal assay	-	low dosage levels	McGregor, 1981
C. Other Indicators of DNA Damaging Potential			
Unscheduled DNA synthesis (UDS) in primary rat liver cells	-	results considered inconclusive because of lack of control data and experimental data	Kramers et al., unpublished; Voogd et al., 1982 (Abstract)
UDS in human diploid fibroblasts	-	large variation among different samples	McGregor, 1981
DNA and hemoglobin alkylation in the mouse	+	guanine-N-7 adducts measured in liver and spleen	Djalali-Behzad et al., 1981

of the inside air by a small electric fan. Petri plates without lids were placed in the container. Methyl bromide was tested at 5 concentrations with S9 mix and 6 concentrations without S9 mix; the type of inducer and species were not specified. A dose response was observed in strain TA100 in the concentration range of 0.5 g/m^3 (0.005 mmol/l) to 5 g/m^3 (0.05 mmol/l) in the absence of S9. At 5 g/m^3, an approximately fivefold increase was observed in numbers of revertants per plate over the background control value. The spontaneous mutant frequency for TA100 was within the normal range. At concentrations higher than 5 g/m^3 the response dropped quickly to zero revertants as toxicity most likely became a factor in the test. Mutagenic activity was similar in the presence and absence of S9 mix. A positive effect was also reported for the base-pair substitution strain TA1535 and _E. coli WP2 hcr_, whereas negative findings were for the frameshift-sensitive strains TA98, TA1538, and TA1537 (data were not reported). Although methyl bromide causes clear mutagenic responses, its activity cannot be compared with the other test agents because its exposure was in the gaseous phase, while the other test agents were added directly to the top agar (i.e., plate incorporation assay).

Methyl bromide (99% purity) and other alkyl halides were evaluated by Simmon (1978, 1981) for mutagenicity in _S. typhimurium_ TA100 without S9 activation. A desiccator procedure was used. Uncovered petri plates inoculated with Salmonella were placed in a gastight chamber. Methyl bromide was injected into the desiccator, and the vapors were dispersed by a fan. Assays were conducted at 37°C for 21 hours. Methyl bromide caused a dose-related positive response at four concentrations ranging from 0.01% to 0.1% in the atmosphere of the desiccators: there were approximately 600 revertants per plate at 0.1%, compared with about 90 revertants per plate in the control. Dichloromethane was mutagenic, whereas carbon tetrachloride was found to be negative in this study by Simmon.

Voogd et al. (1982) and Kramers et al. (1984, in press)*, found methyl bromide (99% purity) to be mutagenic in the bacteria S. typhimurium and K. pneumoniae. Methyl bromide exhibited dose-related mutagenicity in a fluctuation assay for streptomycin resistance using Klebsiella (ur^-, pro^-); the bacteria were incubated for 20 hours at 37°C at three concentrations of methyl bromide in the air of 4.75 g/m^3 (0.05 mmol/l) to 19 g/m^3. Salmonella strains TA98 and TA100 were incubated in closed 1.5-l jars at 37°C for 5 days at 9 concentrations (0 to 50 g/m^3). Dose-related mutagenicity was reported for TA100 at concentrations of 1.9 g/m^3 (0.02 mmol/l) to 19 g/m^3 in the absence of S9 mix (Aroclor 1254-induced rat liver). The maximum response was approximately fourfold over the background counts, which were within the normal range for TA100. At concentrations greater than 19 g/m^3, the mutagenic response dropped because of toxicity. The addition of S9 mix had no effect on the mutagenicity of methyl bromide. However, no postive control requiring S9 activation was reported to insure the S9 was functional. A preincubation assay with Salmonella TA100 showed mutagenicity at 285 mg/l (3 mmol/l) for 6 hours and at 9.50 mg/l for 4 hours. There was no increase in the spontaneous frequency at lower concentrations at these treatment times. A treatment time of 2 hours resulted in no mutagenic effect when tested up to 1000 mg/l. Tester strain TA98 was not reverted by methyl bromide in these studies.

Djalali-Behzad et al. (1981) evaluated methyl bromide (purity not reported, source as a gift) in a forward mutation assay in E. coli Sd-4 (a streptomycin dependent strain). Bacteria were treated in tightly closed tubes for 1 hour at

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*The report by Voogd et al. 1982 is an abstract. A written request was made to obtain the original data. In response to this request, Dr. Voogd sent an unpublished report by Kramer et al. (1984), which has been accepted for publication in Mutation Research (memo to Dr. V. Vaughan-Dellarco).

37°C at 5 concentrations ranging from 0 to approximately 5mM. About a fivefold increase in the mutation frequency was found at 5mM after a 1-hour treatment.

Other bacterial tests in S. typhimurium and E. coli hcr have been reported as positive by Shirasu et al. (1982). Original data were not presented in this article, and the results appeared to be based on those described by Moriya et al. (1983).

4.5.1.2. Drosophila--Kramers et al. (1984, in press; Kramers and Bissumbhar, 1983; Voogd et al., 1982) examined the ability of methyl bromide to cause sex-linked recessive lethals in D. melanogaster. Berlin-K males were treated by inhalation to methyl bromide for 6 hours at 150-750 mg/m^3; 6 hours per day for 5 days at 150-600 mg/m^3; and 6 hours per day, 5 days per week, for 3 weeks at 70-600 mg/m^3. Methyl bromide was lethal at exposures of 600 mg/m^3 for 30 hours or 90 hours. Five broods were examined: males were mated to virgin females every 2 or 3 days for a total period of up to 12 days. Dose-related responses were observed (Table 4-18). A clear, germ-cell stage-specific response was not obtained. The highest lethal frequency, however, was after the 6-hour exposure and was generally observed in brood I, which represents mature sperm. Table 4-18 presents data from the experiment following a 6 hour exposure per day for 5 days at 375 and 487 mg/m^3 methyl bromide. As can be seen from this table, the induced mutation frequency was approximately eightfold and fourteenfold greater than the control. This experiment provides strong evidence that methyl bromide is a mutagen in Drosophila.

McGregor (1981) evaluated methyl bromide (source, BDH Limited, Poole, Dorset, England) in the Drosophila sex-linked lethal test and reported negative results at concentrations of 20 and 70 ppm in air for 5 hours (100 and 350 ppm·h). There were increased responses but they were not dose-response or

TABLE 4-18. Methyl bromide: Drosophila Sex-Linked Resessive Lethal Test

Exposure Time	Exposure Concentration	Number of Lethals/ Number of Tests	% Lethals
5d x 6h	0	2/2320	0.09
	375	17/2339	0.73
	487	16/1282	1.25

SOURCE: Kramers et al., 1984, in press

reproducible. The concentrations evaluated in this study were nontoxic and the sample sizes were small (approximately 1000 chromosomes per dose). Furthermore, negative control data were not given in the study by McGregor. The results of this study are inconclusive.

4.5.1.3. Mammalian Cells in Culture--Kramers et al. (1984, in press) and Voogd et al. (1982) reported methyl bromide to produce a dose-related positive response in gene mutation assays (TK and HGPRT loci) in mouse lymphoma L5178Y cells. Cells were treated in medium for 24 hours at 37°C at five concentrations ranging from 0.3 mg/l (0.003 mmol/l) to 3 mg/l. The mutant frequency in the control culture (treated with 1% ethanol) was 4.6×10^{-5} for TK-deficiency and 1×10^{-5} for HGPRT-deficiency. At the TK locus, the induced mutation frequency was dose-related at five concentrations of methyl bromide. The highest induced mutant frequency, which was observed at 1 mg/l (survival was approximately 60% at this concentration), was approximately a twofold increase in the control frequency. At the next concentration (3 mg/l), the induced mutant frequency dropped slightly as the toxicity increased. At the HGPRT locus, a dose-related response was observed at five concentrations of the chemical. At the highest concentration (3 mg/l), a 14-fold increase was observed over background (survival was approximately 40%).

4.5.2. Chromosomal Aberration Studies

McGregor (1981) examined bone marrow cells from male and female CD rats for chromosomal aberrations after methyl bromide exposure. Animals were exposed to 20 and 70 ppm methyl bromide by inhalation 7 hours per day for 5 consecutive days or for a single exposure of 20 and 70 ppm for 7 hours. Treatment was followed by sampling after 6, 24, and 48 hours. No increases in aberrations were seen after the acute exposures. In the subacute experiments, negative results were obtained except at the 70 ppm for 7 hours per day for 5 days dose for males, where an increase in the frequency of gaps was found. Because gaps are not considered true chromosomal aberrations, their presence alone is insufficient to conclude clastogenic activity. On the other hand, the data do not support a clear negative conclusion because the criteria used to select dosage levels are not stated. It is likely that a maximum tolerated dose was not reached. A toxic dose was apparently not evaluated because the highest exposure (70 ppm) did not affect the rats body weights. The only evidence of adverse effects during or after exposure was traces of blood around the nostrils.

McGregor (1981) conducted a dominant lethal test in male rats exposed to 20 or 70 ppm methyl bromide 7 hours per day for 5 consecutive days. No increase in dominant lethal effects was found. Higher dosage levels should have been examined, especially since the dominant lethal test is not regarded as a sensitive assay (as discussed in section 4.1.2).

4.5.3. Other Studies Indicative of DNA Damage

The potential DNA-damaging activity of methyl bromide was examined in an unscheduled DNA synthesis (UDS) assay referred to as the hepatocyte primary culture DNA repair test (Kramers et al., 1984, in press; Voogd et al., 1982). Hepatocytes were isolated from livers of 6-week-old S.P.F. male Wistar rats.

Cell cultures were incubated with concentrations of methyl bromide (in ethanol) that ranged from 10 mg/l (0.1 mM) to 30 mg/l (0.3 mM) and [^{3}H]thymidine (20 uCi) for 24 hours at 37°C. UDS was measured by autoradiographic determination of the amount of [^{3}H]thymidine that is incorporated into nuclear DNA. For each concentration, nuclear counts are reported after the highest cytoplasmic grain count of three nuclear-sized areas adjacent to the nucleus is subtracted from the nuclear counts (see Williams and Tong, 1980, for details of method). Negative results were reported but no experimental data were presented. The authors' conclusions, therefore, can not be evaluated independently. Also, subtraction of the highest cytoplasmic grain count reduces the possibility of false positives but increases the chance of missing a weak UDS inducer.

McGregor (1981) tested the ability of methyl bromide to induce UDS in human embryonic intestinal cells after exposures of up to 70% in air for 3 hours. The incorporation of [^{3}H]thymidine (10 uCi/ml) was determined by autoradiography. No increase in UDS was detected in the presence or absence of S9 mix (Aroclor 1254-induced rat liver). The positive control, vinyl chloride, induced UDS in the absence of S9 mix; an enhanced response was observed in the presence of S9 mix. High standard deviations, however, were reported among samples; the large variation may be due to the presence of heterogeneous cell populations with different abilities to incorporate [^{3}H]thymidine.

4.5.4. DNA Alkylation Studies

Methyl bromide alkylates DNA in intact mammals (Djalali-Behzad, 1981). DNA alkylation (N-7 guanine) was measured in mouse (male, CBA) liver and spleen after exposure to ^{14}C-labeled methyl bromide (4.9 and 5mCi/mmol) by inhalation for 4 hours. The amount of alkylation was also determined in vitro. Hemoglobin alkylation was measured to determine whether it could be used for an estimation of DNA alkylation in vivo. It was found that the extent of alkylation of DNA in vivo (3.4 x 10^{-9} alkylations/nucleotide at 144 ppm h) was considerably less than the alkylation of hemoglobin in vivo and of DNA and hemoglobin in vitro (Table 4-19).

In a study on the presence of methylated purines in humans after suspected dimethylnitrosamine (DMN) poisoning, DNA samples from the liver and kidney were also examined from one case of methyl bromide poisoning (Herron and Shank, 1980). None of the samples from the methyl bromide poisoning contained 7-methylguanine or O^6-methylguanine, whereas these alkylation products were found in people exposed to DMN. Conclusions cannot be reached from the negative findings on methyl bromide because only one victim was examined, the magnitude of the exposure was not given, and information was not reported on how the tissues were prepared and frozen.

4.5.5. Gonadal Effects

McGregor (1981) examined mice (B6C3F1) for altered sperm morphology after treated exposure to methyl bromide at 20 and 70 ppm by inhalation 7 hours per day for 5 consecutive days. One mouse in 10 died 5 days after exposure. Mice were killed 5 weeks after the last day of treatment. Animals were not sacrificed at different time intervals to examine the effects on various germ-cell stages. Ethyl methanesulfonate (EMS) administered by i.p. injection was the positive control; a gaseous compound given by inhalation would have been more appropriate. There was no evidence of morphological sperm abnormalities induced by methyl bromide. However, these negative findings alone are insufficient to conclude that methyl bromide does not reach the germinal tissues of whole mammals.

4.5.6. Summary and Conclusions

Methyl bromide is mutagenic in bacterial tests using *S. typhimurium*, *E. coli*,

TABLE 4-19. THE ALKYLATION OF CYSTEINE IN HEMOGLOBIN AND LIVER PROTEINS AND OF GUANINE N-7 IN DNA FROM LIVER AND SPLEEN AFTER EXPOSURE OF MICE TO RADIOLABELED METHYL BROMIDE

Administered amount of methyl bromide	Degree of alkylation of cysteine-s		Degree of alkylation of guanine-N-7 in DNA	
(uCi/kg b.w.)	(uCi/g protein) found (expected)[a] in		(uCi/gDNA) found (expected)[a] in	
	Hb	Liver	Liver	Spleen
340 (inhalation)	2.2×10^{-2}	1×10^{-3} $(>2.2 \times 10^{-2})$	5×10^{-5} (1×10^{-2})	5×10^{-4} (1×10^{-2})
174 (inhalation)	1.6×10^{-2}			
21.6 (i.p. injection)	4.2×10^{-3}			

[a] Expected from the reactivities in vitro of cysteine-S in hemoglobin and guanine-N-7 in DNA, respectively, assuming that the dose of methyl bromide is equal to the dose obtained in red blood cells.

SOURCE: Djalali-Behzad et al., 1981.

and *K. pneumoniae*. Clear dose-dependent mutagenic responses have been reported. The mutagenicity of methyl bromide is consistent with its alkylating properties in that it is a direct-acting mutagen and induces primarily base-pair substitution mutations. The mutagenic responses were not enhanced by an S9 metabolic activation system.

In addition to its being a bacterial mutagen, methyl bromide is reported to be mutagenic in eucaryotic organisms. It has been shown to induce gene mutations in Drosophila and at two different loci in mammalian cells in culture. The positive findings in bacteria, cultured mammalian cells, and Drosophila, coupled with the ability of methyl bromide to alkylate DNA in intact mammals is sufficient evidence to presume that methyl bromide has the potential to be mutagenic in whole mammals. Methyl bromide does not appear to be as potent a mutagen as ethylene dibromide in that more chemical is needed to induce a response. Data regarding its ability to cause chromosomal aberrations have been primarily negative. However, these results appear to have been generated using inadequate exposure levels. Furthermore, chromosome tests are generally not as sensitive as gene mutation tests (Voogd et al., 1977). Although, methyl bromide does not appear to elicit UDS, the testing has been limited and inadequate. Moreover, certain kinds of DNA lesions that lead to mutations may not stimulate the repair process or do so to such a small extent that UDS would not be detected. Methyl bromide's potential to reach the germinal tissue of intact mammals has not been sufficiently investigated; one test for altered sperm morphology and a test for dominant lethal effects have been reported as negative. In view of the fact that methyl bromide induces heritable effects in the germ cells of Drosophila, it should be further evaluated for such effects in mammalian germ cells.

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